UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

EFEITOS DO *DIABETES MELLITUS* E DA SUPLEMENTAÇÃO COM ANTIOXIDANTES NO ESTRESSE OXIDATIVO E NA EXPRESSÃO DE MIOSINAS NO CÉREBRO DE RATOS

Aluno: Luciana Karen Calábria

Orientador: Prof. Dr. Foued Salmen Espindola

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ALUNO: Luciana Karen Calábria

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Prof. Dr. Foued Salmen Espindola

"Se você quer avançar para o infinito
explore o finito em todas as direções."
Goethe

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APRESENTAÇÃO

O formato desta tese obedece às normas do Programa de Pós-graduação em Genética e Bioquímica. Ela é composta de três capítulos, sendo o capítulo 1 referente à fundamentação teórica, que embasa os outros dois capítulos.

Capítulo 1 - Fundamentação Teórica.

Capítulo 2 - Superexpressão de miosina-IIB no cérebro de ratos diabéticos induzidos por estreptozotocina.

Capítulo 3 – Efeito da suplementação de cálcio, zinco e vitamina E no estresse oxidativo e na expressão de miosinas no cérebro de ratos diabéticos induzidos por estreptozotocina.

Os capítulos 2 e 3 foram escritos no formatado de um artigo científico, em inglês, revisado por nativos que compõem a Comissão do *American Journal Experts* (*http://www.journalexperts.com*). Cada artigo representa o estudo na íntegra, está formatado dentro das normas das revistas citadas nos seus referentes capítulos, e após as considerações dos membros da banca e a defesa da tese foram submetidos para publicação.

Capítulo 1

1. FUNDAMENTAÇÃO TEÓRICA

1.1 *Diabetes mellitus*

Segundo a Sociedade Brasileira de Diabetes, o *Diabetes mellitus* é uma doença metabólica caracterizada pelo aumento significativo de glicose circulante no sangue, resultante de anormalidades na secreção e/ou ação da insulina, tendo como consequência os distúrbios no metabolismo de carboidratos, gorduras e proteínas. Em países em desenvolvidos, esta doença é a maior causa de mortalidade (Tunali e Yanardag, 2006) e vem aumentando consideravelmente o seu acometimento na população mundial, sendo um significante fator de risco para problemas vasculares (Watkins, 2003) e complicações que afetam os olhos, rins, coração, nervos e o cérebro (Mccall, 1992; Biessels, Kappelle *et al.*, 1994; Gispen e Biessels, 2000).

A insulina é um hormônio secretado pelas células beta das ilhotas de Langerhans do pâncreas, responsável pela captação e direcionamento da glicose livre na corrente sanguínea para os tecidos. Além de controlar a glicemia, a insulina é importante na estimulação da síntese protéica e no controle do peso corporal, atuando como fator de crescimento e diferenciação celular, incluindo os neurônios do sistema nervoso central, como também participando dos processos de formação de memória, aprendizado e plasticidade sináptica (Zhao, Chen *et al.*, 1999; Mauvais-Jarvis e Kahn, 2000; Haber, Curi *et al.*, 2001; Park, 2001).

A glicose é a principal fonte de energia do organismo, essencial para o corpo na realização de suas funções, como crescimento, reparo, atividade física e manutenção da temperatura corporal. Porém, quando em excesso, pode trazer várias complicações à saúde. No sistema nervoso central, os distúrbios vão desde alterações na neurotransmissão e nos níveis de neurotransmissores, mudanças estruturais, redução na atividade motora, comportamento depressivo, morte celular, até anormalidades eletrofisiológicas e na aprendizagem e memória (Lackovic, Salkovic *et al.*, 1985; Bitar, Koulu *et al.*, 1987; Mooradian, 1988; Mccall, 1992; Biessels, Kappelle *et al.*, 1994; Di Mario, Morano *et al.*, 1995; Helkala, Niskanen *et al.*, 1995; Ramakrishnan, Suthanthirarajan *et al.*, 1996; Ramakrishnan, Nazer *et al.*, 2003; Ramakrishnan, Sheeladevi *et al.*, 2004;

Ramakrishnan, Prabhakaran *et al.*, 2005; Hernandez-Fonseca, Rincon *et al.*, 2009).

1.2 Diabetes e o estresse oxidativo

No *diabetes mellitus,* a hiperglicemia é responsavel por inúmeros efeitos sobre a célula e seus constituintes, provocando alterações celulares decorrentes do processo de glicação não-enzimática e da glico-oxidação (Baynes e Thorpe, 1999). Estes processos estão relacionados com o aumento na produção de espécies reativas do oxigênio (Figura 01) e com a formação de produtos finais da glicação avançada, os quais contribuem para a modificação irreversível de proteínas, DNA e lipídios; e com o aumento da presença de produtos de peroxidação lipídica (Jennings, Jones *et al.*, 1987; Rosen, Nawroth *et al.*, 2001; Genet, Kale *et al.*, 2002; Siddiqui, Taha *et al.*, 2005). Além disso, esse processo em que a glicose é oxidada na presença de íons metálicos livres leva a liberação de radicais superóxido e hidroxila, que pode afetar a oxidação de proteínas (Wolff e Dean, 1987; Wolff, Jiang *et al.*, 1991).

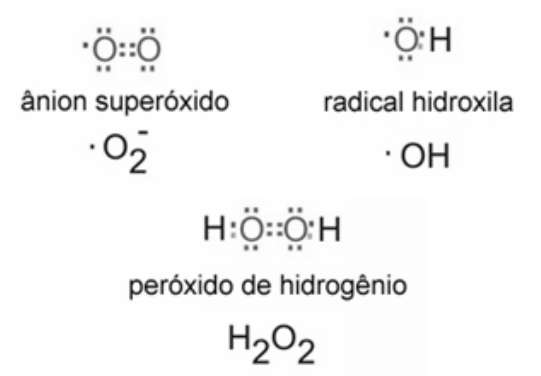

Figura 01: Espécies reativas do oxigênio. Radical ânion superóxido, radical hidroxila e não-radical peróxido de hidrogênio.

Vários estudos têm voltado a atenção no estado antioxidante e no aumento do estresse oxidativo no diabetes (Uzel, Sivas *et al.*, 1987; Jennings, 1994; Oranje, Rondas-Colbers *et al.*, 1999). Entretanto, ainda não há um consenso se este aumento é o fator primário nas complicações desta doença, ou se este é meramente consequência dos danos teciduais, refletindo a presença das

complicações, que podem ser explicadas por inúmeras hipóteses, como: 1) o aumento do fluxo pela via dos polióis, aumentando o estresse oxidativo (Lee e Chung, 1999); 2) o aumento dos produtos finais da glicação avançada, e de glicação de proteínas da membrana plasmática e da matriz extracelular (Wautier, Wautier *et al.*, 1994); 3) a ativação da via da proteína quinase C, gerando aumento de citocinas, estresse oxidativo e fatores proliferativos (Ishii, Koya *et al.*, 1998); e 4) o aumento do fluxo pela via das hexosaminas, que também levaria a um aumento de citocinas (Sharma e Ziyadeh, 1997), como está apresentado na figura 02.

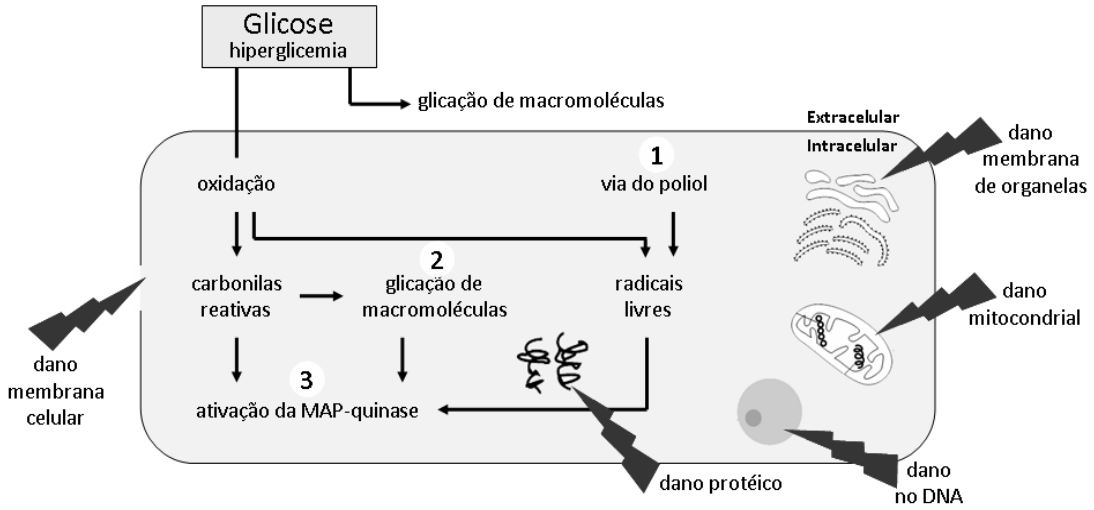

Figura 02: Esquema representativo das consequências bioquímicas e morfológicas no interior celular devido a alta concentração de glicose circulante.

Por outro lado, a atividade das enzimas antioxidantes das espécies reativas, incluindo a glutationa, catalase e superóxido dismutase, pode ser afetada durante o diabetes (Wohaieb e Godin, 1987; Genet, Kale *et al.*, 2002). Enquanto o aumento de glicação de proteínas pode levar à redução na atividade da enzima superóxido dismutase (Mohammad, Taha *et al.*, 2004; Siddiqui, Taha *et al.*, 2005), a alteração nos níveis de glutationa pode estar relacionada com o aumento da via do poliol (Preet, Gupta *et al.*, 2005), levando a depleção de NADPH (nicotinamida adenina dinucleotídeo fosfato) que é necessário para a redução da glutationa oxidada. A diminuição local de compostos antioxidantes endógenos pode ser

devido ao aumento do consumo pelas espécies reativas, elevando a quantidade de peróxido de hidrogênio (Ikebuchi, Kashiwagi *et al.*, 1993).

O aumento da peroxidação lipídica já foi demonstrado no cérebro de ratos diabéticos e ratos envelhecidos (Mooradian e Smith, 1992; Kumar e Menon, 1993; Leutner, Eckert *et al.*, 2001; Genet, Kale *et al.*, 2002; Siddiqui, Taha *et al.*, 2005; Sinha, Baquer *et al.*, 2005; Kumar, Taha *et al.*, 2008). Os produtos oriundos da oxidação de ácidos graxos insaturados e do colesterol podem ser mensurados para avaliar a peroxidação lipídica que ocorre nos tecidos. Dentre estes produtos, o aldeído é um dos mais estudados (Esterbauer, Gebicki *et al.*, 1992). Diversos aldeídos reativos, como o malondialdeído, podem se ligar aos resíduos de aminoácidos positivamente carregados das apolipoproteínas, principalmente da lisina, produzindo alterações de cargas na superfície das lipoproteínas (Holvoet, Perez *et al.*, 1995; Kesavulu, Rao *et al.*, 2001).

1.3 Cérebro e o estresse oxidativo

O cérebro é especialmente susceptível ao dano oxidativo devido: 1) ao consumo elevado de oxigênio, ATP e glicose; 2) ao seu abundante conteúdo lipídico; 3) ao líquido cefalorraquidiano conter complexos de ferro e cobre, que catalisam a formação de radicais hidroxila altamente reativos; 4) à liberação de espécies reativas do oxigênio durante a oxidação da dopamina e neurotransmissão glutamatérgica; 5) à interação do óxido nítrico com o radical superóxido, levando a degeneração neuronal; 6) à baixa quantidade relativa de enzimas antioxidantes comparado com outros tecidos; 7) aos neurônios serem células não-replicáveis e qualquer dano ao tecido cerebral pelas espécies reativas do oxigênio ser acumulativo ao longo do tempo (Sacks, 1965; Olanow, 1993; Reiter, 1995; Halliwell, 2001; Cui, Luo *et al.*, 2004; Poon, Vaishnav *et al.*, 2006; Yanardag e Tunali, 2006; Baquer, Taha *et al.*, 2009; Nazaroglu, Sepici-Dincel *et al.*, 2009). Além disso, vários estudos demonstram que o diabetes está associado com um aumento nos danos cerebrais provocados pelo estresse oxidativo no cérebro (Sanders, Rauscher *et al.*, 2001; Ozkaya, Agar *et al.*, 2002; Yanardag e Tunali, 2006; Celik e Erdogan, 2008; Nazaroglu, Sepici-Dincel *et al.*, 2009). Em diabéticos, os níveis de glicose são responsávéis pelo aumento de até quatro

vezes nos níveis de glicose neuronal. Se isso é persistente ou se torna um evento regular, o metabolismo da glicose intracelular pode levar a um desbalanço entre a defesa antioxidante e os danos teciduais no cérebro, assim como nos neurônios (Pari e Latha, 2004; Tomlinson e Gardiner, 2008). Além disso, a hiperglicemia pode causar o aumento na produção de radicais livres via auto-oxidação da glicose e glicação enzimática de proteínas, levando ao dano oxidativo nas membranas (Wolff, 1993), ativando a apoptose celular e alterando a transmissão sináptica (Arroba, Frago *et al.*, 2005; Artola, 2008; Tomlinson e Gardiner, 2008).

Estudos sugerem que os radicais livres derivados (radicais superóxido, peróxido e hidroxila) desempenham papel crucial no diabetes, promovendo a glicação não-específica de proteínas, peroxidação de lipídios em membranas, interação de proteínas, deficiência na função de organelas e morte celular. No entanto, o sistema biológico possui mecanismos de defesa contra essas espécies reativas do oxigênio, sendo que em condições fisiológicas normais, existe um balanço entre a produção de espécies reativas do oxigênio e os sistemas antioxidantes (Taniyama e Griendling, 2003; Robertson, Harmon *et al.*, 2004).

1.4 Antioxidantes

O organismo possui sistemas de defesa antioxidante enzimático e não-enzimático (Figura 03) que incluem as moléculas que estabilizam as espécies reativas do oxigênio, ácido úrico, ácido ascórbico, alfa-tocoferol; moléculas que contêm sulfidrila e enzimas antioxidantes, como o superóxido dismutase, a catalase e a glutationa peroxidase (Frei, Stocker *et al.*, 1988; Stinefelt, Leonard *et al.*, 2005). Em condições patológicas, em que a produção excessiva de espécies reativas do oxigênio supera a defesa antioxidante, o estresse oxidativo pode modificar irreversivelmente macromoléculas biológicas, como o DNA, as proteínas, os carboidratos e os lipídeos (Du, Edelstein *et al.*, 2000).

Considerando que o estresse oxidativo desempenha um importante papel nas complicações do diabetes, a terapia antioxidante tem atraído a atenção de pesquisadores. Além dos estudos com antioxidantes tradicionais, como vitamina C, vitamina E e superóxidos miméticos, outras moléculas com ação antioxidante têm sido investigadas. Vários estudos demonstram o papel de macronutrientes na

prevenção do diabetes (Marshall, Hoag *et al.*, 1994; Meyer, Kushi *et al.*, 2000; Van Dam, Willett *et al.*, 2002; Liu, Serdula *et al.*, 2004; Schulze, Liu *et al.*, 2004; Tinker, Bonds *et al.*, 2008), mas, por outro lado, existem poucas evidências do papel das vitaminas e minerais na prevenção primária e secundária desta doença. Sendo assim, a justificativa para a utilização destes antioxidantes baseia-se em grande parte, nas experiências com animais e em estudos epidemiológicos (Kadowaki e Norman, 1984; Beaulieu, Kestekian *et al.*, 1993; Feskens, Virtanen *et al.*, 1995; Will, Ford *et al.*, 1999; Maestro, Campion *et al.*, 2000; Ford e Mokdad, 2001; Pittas, Lau *et al.*, 2007).

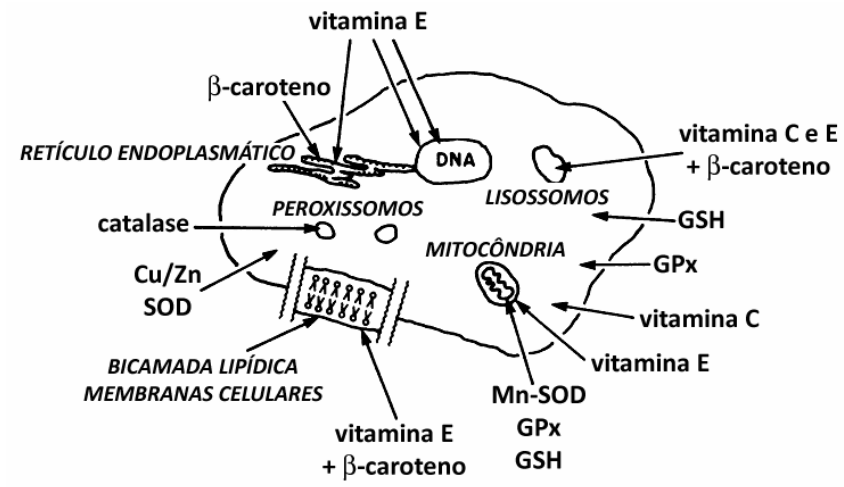

Figura 03: Origem dos antioxidantes celulares. Modificado de Machlin e Bendich, 1987.

Como o diabetes está associado com o aumento do estresse oxidativo (Wen, Skidmore *et al.*, 2002; Ceriello e Motz, 2004), este fato reforça o interesse no uso de suplementos antioxidantes como uma tentativa de prevenir as complicações a longo prazo. No que diz respeito à prevenção do diabetes e a modificação na dieta, os relatos atuais ainda não permitem que qualquer recomendação segura e específica seja feita em relação ao uso de suplementos. Dado que o diabetes é uma condição de estresse oxidativo aumentado, a terapia antioxidante poderia representar um potencial coadjuvante no tratamento farmacológico antidiabético.

Apesar do uso de suplementos oferecer benefícios aparentes, ainda são necessários dados mais consistentes sobre os efeitos benéficos em relação ao

diabetes. Quanto às altas doses de suplementos antioxidantes, já existem dados que indicam não só a falta do benefício em termos do controle glicêmico, mas também a progressão das complicações do diabetes e de danos potenciais (Halliwell, 1995; Hasanain e Mooradian, 2002; Ward, Wu *et al.*, 2007).

1.4.1 Antioxidantes enzimáticos

As enzimas antioxidantes têm como principal função eliminar as espécies reativas do oxigênio e corrigir pequenos desvios nas concentrações fisiológicas destas moléculas. As alterações na atividade destas enzimas podem ser consideradas como biomarcadores da resposta antioxidante (Sies, 1993). Neste contexto, temos três enzimas: superóxido dismutase, catalase e glutationa peroxidase (Figura 04).

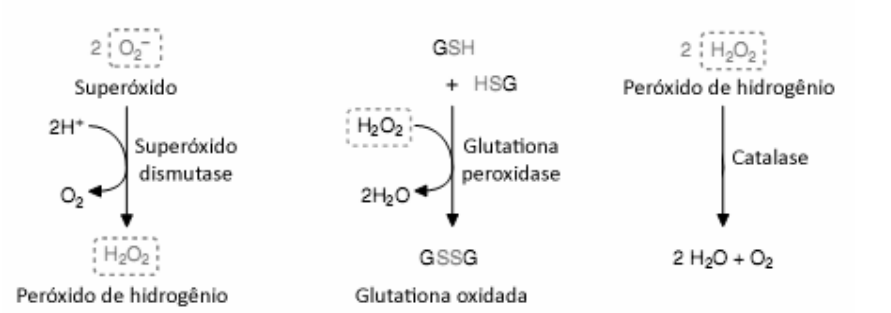

Figura 04: Antioxidantes enzimáticos.

Os processos de formação do superóxido e do peróxido estão correlacionados, pois o superóxido é convertido em peróxido e oxigênio por uma reação catalisada pela superóxido dismutase. Esta enzima possui várias isoformas, diferindo-se quanto à natureza do centro metálico ativo, por sua constituição em aminoácidos, pelo número de subunidades, pelos seus cofatores e outras características. Os efeitos citotóxicos do peróxido de hidrogênio são limitados por sua degradação pela catalase. Esta enzima, por sua vez, exerce a função de decompor o peróxido de hidrogênio em água e oxigênio, existindo sob duas isoformas: selênio-independente e selênio-dependente (Mates, Perez-Gomez *et al.*, 1999), que diferem-se quanto ao número de subunidades, a natureza ligante do selênio no centro ativo e quanto aos seus mecanismos catalíticos. O substrato para a reação catalítica da glutationa peroxidase é o

peróxido de hidrogênio ou o peróxido orgânico, que são decompostos em água ou álcool. A glutationa peroxidase compete com a catalase pelo peróxido de hidrogênio como substrato e é a principal fonte de proteção contra as espécies reativas do oxigênio (Valko, Rhodes *et al.*, 2006).

Em diabéticos, as atividades da superóxido dismutase e da catalase, parecem estar diminuídas no cérebro de ratos (Kumar e Menon, 1993; Makar, Rimpel-Lamhaouar *et al.*, 1995); enquanto que no cérebro de camundongos foi observado o aumento na atividade desta enzima (Huang, Juang *et al.*, 1999). Isso demonstra o quanto os resultados referentes ao estresse oxidativo são variáveis entre as espécies.

1.4.2 Antioxidantes não-enzimáticos

Vários compostos não-enzimáticos, como a glutationa reduzida, incluindo os carotenóides e as vitaminas A, C e E, têm sido relatados por possuírem propriedade antioxidante no plasma e em tecidos (Frei, Stocker *et al.*, 1988), além de alguns minerais, como zinco, magnésio e selênio (Martini, Catania *et al.*, 2010).

Os tocoferóis são chamados antioxidantes primários porque interrompem diretamente a oxidação, convertendo os radicais livres em espécies mais estáveis. A vitamina E (tocoferol-OH) é um clássico exemplo de antioxidante que limita os efeitos deletérios das reações oxidantes, interrompendo as reações em cadeia iniciadas pelos radicais livres, doando um átomo de hidrogênio para um radical peroxil para formar peróxido lipídico, impedindo o dano oxidativo (Burton e Traber, 1990; Martini, Catania *et al.*, 2010). Contudo, quando a vitamina E age, são gerados radicais tocoferoxila e a regeneração é requerida, a fim de evitar indesejáveis processos oxidativos mediados pela tocoferoxila. Assim, os resultados contraditórios de estudos com vitamina E, podem ser devido à falta de avaliação deste sistema de regeneração, composta de ácido ascórbico, glutationa reduzida e co-enzima Q10 (Nwose, Jelinek *et al.*, 2008).

Estudos recentes de diabetes em animais mostram que o uso da vitamina E reduz o risco da doença e suas complicações (Sena, Nunes *et al.*, 2008; Shirpoor, Salami *et al.*, 2009), inclusive tendo efeito protetor no cérebro (Kabay,

Ozden *et al.*, 2009). No entanto, uma importante limitação destes estudos observacionais é que não se consegue distinguir claramente se o menor risco da doença, associado com os altos níveis de vitamina E, é devido à suplementação da vitamina ou a outros fatores ligados ao estilo de vida, tais como o aumento da prática de exercício físico e uma dieta mais saudável. Sendo assim, esses ensaios clínicos não confirmam os benefícios da vitamina E, de forma isolada, na prevenção e/ou tratamento do diabetes.

O zinco não é considerado um antioxidante como a vitamina E. Entretanto, este mineral pode limitar os danos induzidos pelo estresse oxidativo (Bunk, Dnistrian *et al.*, 1989; Noh e Koo, 2001), estabilizando a estrutura da membrana plasmática (Bray e Bettger, 1990), restringindo a produção de radicais livres endógenos (Bray e Bettger, 1990; Bell, Sakanashi *et al.*, 1998), contribuindo na estrutura da enzima superóxido dismutase (Marklund, 1982; Davis, Klevay *et al.*, 1998), e mantendo a concentração de metalotioneína tecidual, também considerado um protetor contra os danos oxidativos (Cousins, 1985; Ebadi, Leuschen *et al.*, 1996; Rojas, Cerutis *et al.*, 1996; El Refaey, Ebadi *et al.*, 1997). Além disso, em vários sistemas, o zinco pode antagonizar a propriedade catalítica dos metais de transição, ferro e cobre, no que diz respeito à sua capacidade de promover a formação de hidroxilas pelo peróxido de hidrogênio e superóxido (Powell, 2000).

No cérebro de diabéticos, estudos mostraram que o tratamento com zinco reduz significativamente a astrocitose (Wei, Liu *et al.*, 2009) e previne a apoptose induzida (Thomas e Caffrey, 1991; Matsushita et al., 1996) sugerindo ser um tratamento potencial na prevenção dos efeitos deletérios do diabetes e na redução do estresse oxidativo (Santon, Formigari *et al.*, 2006). Por outro lado, outros estudos comprovaram que a suplementação de zinco induz à apoptose (Weissgarten, Berman *et al.*, 2002; Chang, Torzillo *et al.*, 2006). Wiseman, Wells *et al.*, (2007) revelaram que o aumento de zinco intracelular é um evento chave associado à interrupção da função mitocondrial, além de induzir vias apoptóticas mediadas pelo peróxido de hidrogênio. Além do mais, existem evidências de que a ingestão excessiva de zinco pode induzir a uma condição patológica associada ao estresse oxidativo (Yanagisawa, Sato *et al.*, 2004).

Estudos clínicos sugerem que a suplementação com o cálcio pode afetar indiretamente o metabolismo da glicose (Zemel, Thompson *et al.*, 2004; Zemel, Donnelly *et al.*, 2008) prevenindo o *Diabetes mellitus* do tipo II. Desta forma, o potencial papel da suplementação de cálcio na prevenção primária do diabetes tem sido investigado (Pittas, Lau *et al.*, 2007; De Boer, Tinker *et al.*, 2008).

1.5 Cálcio/Calmodulina

Ambos os diabetes, tipo I e tipo II, estão associados com os distúrbios na regulação do cálcio intracelular. A hiperglicemia causa uma mudança nos níveis de cálcio citosólico devido a um aumento no influxo deste íon e a mobilização do mesmo no interior de algumas células, promovendo a estocagem. Além disso, a hiperglicemia tem sido associada a um decréscimo do efluxo de íons cálcio da célula (Massry e Smogorzewski, 1997). A combinação do aumento do influxo e o decréscimo do efluxo de cálcio leva a um aumento nos níveis basais citosólicos que pode, consequentemente, afetar a função celular (Figura 05). Deste modo, o aumento de cálcio citosólico durante o diabetes está ligado à falha de vários componentes de transdução de sinal podendo levar a um prejuízo neuronal associado com o estado patológico, aumentando a morte celular em áreas específicas do cérebro (Klein, Hains *et al.*, 2004), e podendo alterar os níveis e a função de proteínas ligantes de cálcio/calmodulina.

O aumento da entrada de cálcio pode acontecer devido à ativação de canais de cálcio mediado pela proteína-G, levando a estimulação de várias vias celulares, incluindo a via da proteína quinase dependente de AMPc e canais de cálcio, o sistema da proteína quinase e o da fosfolipase C, estabelecendo um novo processo patológico sujeito à disfunção celular durante as condições hiperglicêmicas (Demerdash, Seyrek *et al.*, 1996; Massry e Smogorzewski, 1997). Por outro lado, Bhardwaj e Kaur (1999) demostraram em diabetes, um aumento da atividade do AMPc e da proteína kinase A, e uma diminuição da atividade da fosfolipase A_2 em terminações nervosas, que são mediadas pela ativação da proteína quinase II dependente de cálcio/calmodulina (CaMKII) (Piomelli e Greengard, 1991).

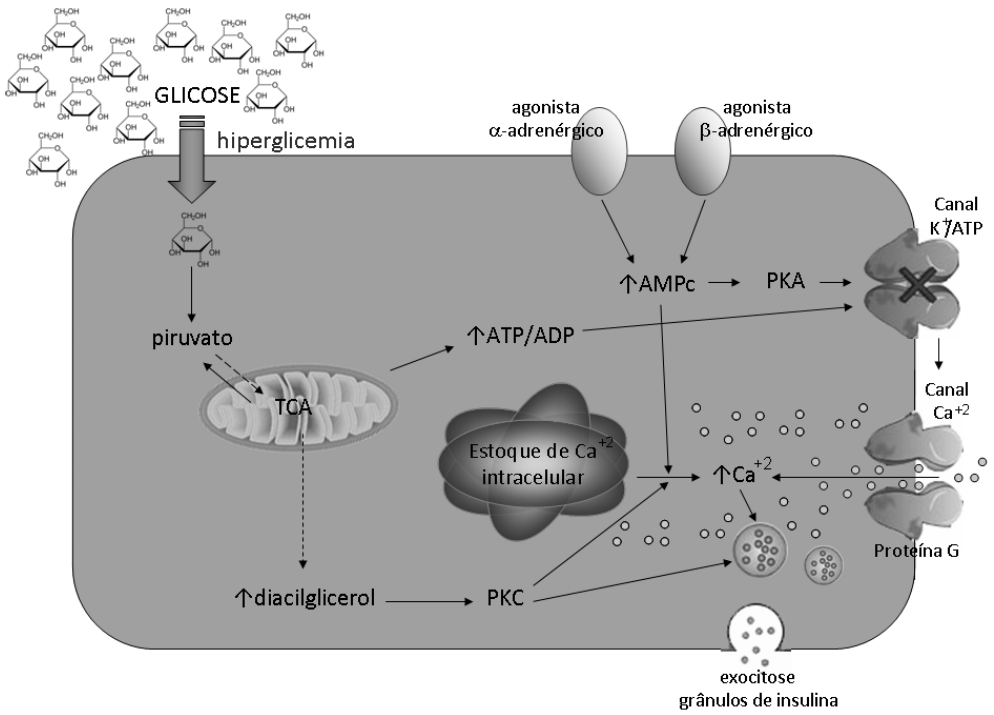

Figura 05: Vias bioquímicas intracelulares afetadas pelo aumento no influxo de íons cálcio.

A calmodulina é uma proteína relativamente pequena, com aproximadamente 149-155 aminoácidos e massa molecular de aproximadamente 16 kDa, dependendo do organismo (Polans, Baehr *et al.*, 1996). É uma proteína ligante de cálcio e a análise da sua estrutura tridimensional mostra dois domínios globulares conectados por uma alfa-hélice central onde residem motivos *helix-loop-helix* (EF-hands), que se ligam por afinidade ao cálcio (Bhattacharya, Bunick *et al.*, 2004) (Figura 06).

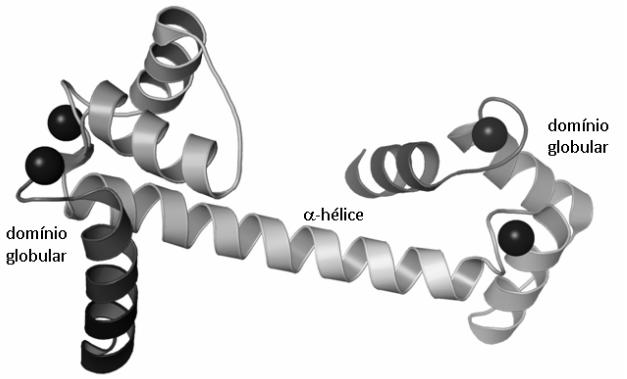

Figura 06: Estrutura tridimensional da calmodulina. Modificado de (Bhattacharya, Bunick *et al.*, 2004).

A comparação da estrutura e função da calmodulina em diferentes organismos indica que essa molécula é altamente conservada (Waisman, Stevens *et al.*, 1975; Dedman, Welsh *et al.*, 1978; Krebs, 1981), se alterando pouco durante a evolução, embora algumas espécies possuam mais de uma isoforma. Uma prova disto é a variação em um pequeno número de aminoácidos funcionalmente idênticos (Klee, Draetta *et al.*, 1988) entre a molécula de mamífero e a de microorganismos eucarióticos.

A calmodulina está diretamente relacionada com a secreção de insulina no pâncreas (Norling, Colca *et al.*, 1994; Matsumoto, Fukunaga *et al.*, 1995; Ribar, Epstein *et al.*, 1995), afetando os alvos de insulina em tecidos, incluindo o fígado, coração, tecido adiposo, rim e músculo esquelético (Morley, Levine *et al.*, 1982; Hoskins e Scott, 1983; Solomon, Palazzolo *et al.*, 1990; Ozturk, Aydin *et al.*, 1994; Solomon, Palazzolo *et al.*, 1994).

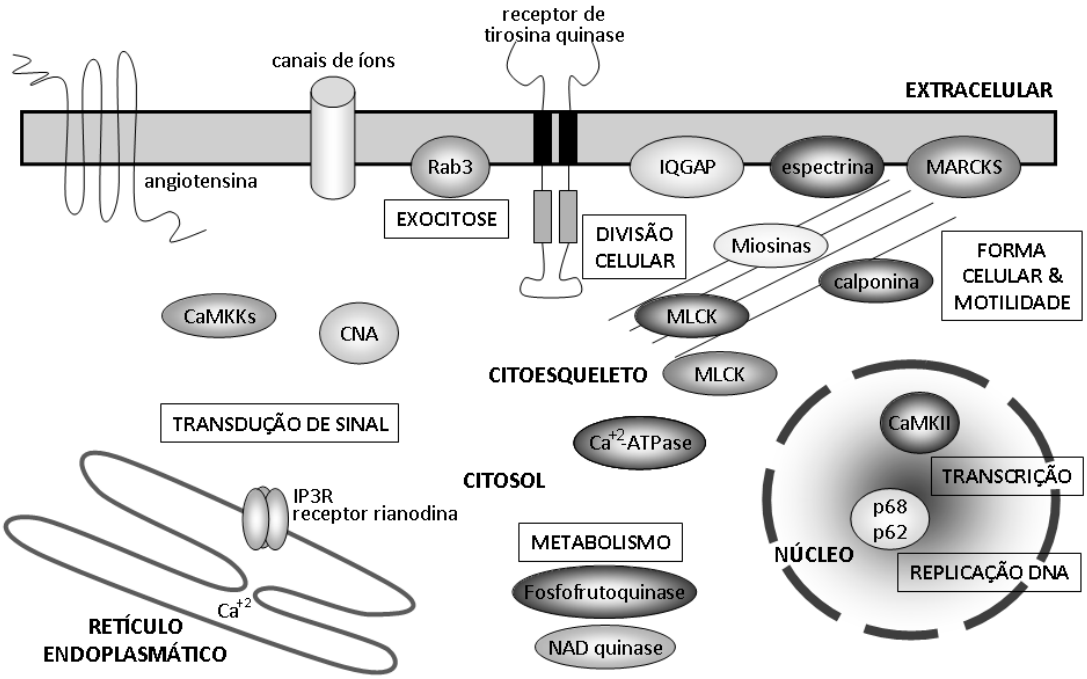

Figura 07: Algumas proteínas ligantes de calmodulina com sua localização celular e função. Modificado de (O'day, 2003).

A ligação de cálcio à calmodulina causa uma mudança conformacional na molécula desta proteína, de tal maneira que o complexo cálcio/calmodulina interage e regula várias enzimas e proteínas-alvo envolvidas em diferentes

aspectos da atividade celular (Figura 07), como síntese e degradação de nucleotídeos, transcrição de genes, regulação de diferentes sistemas de transporte, controle do metabolismo celular, organização do citoesqueleto, citocinese, contração muscular, regulação do volume osmótico, endocitose e exocitose, fertilização do zigoto, comunicação intercelular, proliferação celular, diferenciação e apoptose (Cheung, 1980; Klee e Vanaman, 1982; Means, Tash *et al.*, 1982; Carafoli, 1987; Babu, Bugg *et al.*, 1988; Espindola, Espreafico *et al.*, 1992; Chamberlain, Roth *et al.*, 1995; Carafoli, Nicotera *et al.*, 1997; Colombo, Beron *et al.*, 1997; Chin e Means, 2000; Carafoli, Santella *et al.*, 2001).

1.6 Proteínas ligantes de cálcio/calmodulina

As proteínas ligantes de calmodulina compreendem um grupo diversificado. Essa interação é regulada usualmente pelo nível citoplasmático de íons cálcio e baseado nisso é possível que as proteínas ligantes se classifiquem em três categorias: Ca^{+2}-dependente, Ca^{+2}-independente e Ca^{+2}-inibido (O'day, 2003). Algumas proteínas Ca^{+2}-dependentes têm um ou mais domínios ligantes de calmodulina com aproximadamente 20 resíduos de aminoácido, e têm sido agrupadas em dois motivos relacionados, baseados na posição dos resíduos hidrofóbicos conservados (Crivici e Ikura, 1995; Rhoads e Friedberg, 1997), como 1-8-14 (Dasgupta, Honeycutt *et al.*, 1989) e 1-5-10 (Picciotto, Czernik *et al.*, 1993). Por outro lado, a calmodulina também pode se ligar às proteínas-alvo de maneira Ca^{+2}-independente através de uma sequência repetida de isoleucina e glutamina (IQxxxRGxxxR), também chamada de motivo IQ. Em algumas regiões do cérebro de rato existem poucas proteínas ligantes de calmodulina Ca^{+2}-independentes, mas um grande número de proteínas Ca^{+2}-dependentes (O'day, Lydan *et al.*, 2001; O'day, Payne *et al.*, 2001).

Dentre as proteínas ligantes de calmodulina, tem-se a CaMKII que é o principal mediador neuronal de sinalização via cálcio, integrando múltiplas funções relacionadas. Esta molécula, que parece ser relativamente vulnerável em estágios patológicos, está associada com o influxo de cálcio dentro da célula e está implicada numa variedade de eventos em neurônios, como na liberação e

síntese de neurotransmissores e canais iônicos, e na expressão gênica (Bading, Ginty *et al.*, 1993; Kitamura, Miyazaki *et al.*, 1993; Blanquet e Lamour, 1997).

A literatura revela a isquemia e a hipoglicemia associadas a uma modulação permanente da atividade da CaMKII (Hu, Kurihara *et al.*, 1995; Hu e Wieloch, 1995; Kolb, Hudmon *et al.*, 1995), mostrando que a elevação dos níveis de cálcio intracelular em cultura de neurônios resulta na autofosforilação da CaMKII e produz a forma da enzima independente de cálcio (Fukunaga, Rich *et al.*, 1989; Fukunaga, Soderling *et al.*, 1992). Em um estado hiperglicêmico, os níveis da CaMKII e a sua atividade estão aumentados em diferentes regiões do cérebro (Bhardwaj e Kaur, 1999). As alterações drásticas da atividade desta enzima podem ser atribuídas aos fatores de modificação covalente ou interações endógenas do inibidor/ativador. No entanto, é também sugerido que o mecanismo que envolve a CaMKII em funções neuronais esteja relacionado com a regulação da expressão gênica.

Berggard, Arrigoni *et al.*, (2006) identificaram 18 proteínas ligantes de calmodulina em cérebro de camundongo envolvidas em função do citoesqueleto, como actina, dineína, mielina, espectrina e tubulina. Além destas proteínas do citoesqueleto e motores moleculares, algumas miosinas também se ligam a calmodulina pelo motivo IQ (Hoyt, Hyman *et al.*, 1997).

1.7 Miosinas

A superfamília miosina, baseado em análise do domínio motor, possui pelo menos 20 classes (Berg, Powell *et al.*, 2001; Krendel e Mooseker, 2005), apesar de dados filogenéticos revelarem pelo menos 35 classes (Odronitz e Kollmar, 2007), e por análises do sequenciamento genômico cerca de 40 classes de miosinas (Richards e Cavalier-Smith, 2005).

As miosinas são proteínas motoras conservadas, encontradas em todos os eucariotos de levedura a mamíferos, possuindo atividade ATPase que converte a energia de hidrólise da adenosina trifosfato (ATP) em movimento quando ligada à actina. Muitas das cadeias pesadas de miosinas consistem de três domínios: 1) a cabeça globular N-terminal ou domínio motor catalítico, que possui sítios ligantes de ATP e actina; 2) região do pescoço, onde se ligam as cadeias leves de miosina

e/ou calmodulina, consistindo de um ou mais motivos IQ; 3) e uma cauda C-terminal, capaz de se ligar às cargas que serão transportadas ou ainda interagir com o domínio cauda de outras miosinas. Vários estudos têm indicado este domínio como o mais divergente entre as classes de miosinas, o que confere diferentes funções celulares a estes motores moleculares (Hoyt, Hyman *et al.*, 1997; Buss, Spudich *et al.*, 2004; Krendel e Mooseker, 2005).

Embora as miosinas desempenhem diversos papéis, incluindo o movimento de organelas, endocitose, exocitose, transporte de RNAm e transdução de sinal em diferentes tipos celulares (Titus, 1997; Mermall, Post *et al.*, 1998) (Figura 08), somente as das classes I, II, V, VI, IX e XVIII participam de funções específicas nos neurônios de vertebrados (Bridgman e Elkin, 2000; Bridgman, 2004), estando também envolvidas em patologias, como a miosina-II em miopatias (Seidman e Seidman, 2001), perda de audição (Avraham, 2002) e macrotrombocitopenia (Heath, Campos-Barros *et al.*, 2001), e a miosina-V na Síndrome de Griscelli (Pastural, Barrat *et al.*, 1997; Westbroek, Lambert *et al.*, 2001) e hipotiroidismo (De Souza Martins, Romao *et al.*, 2009).

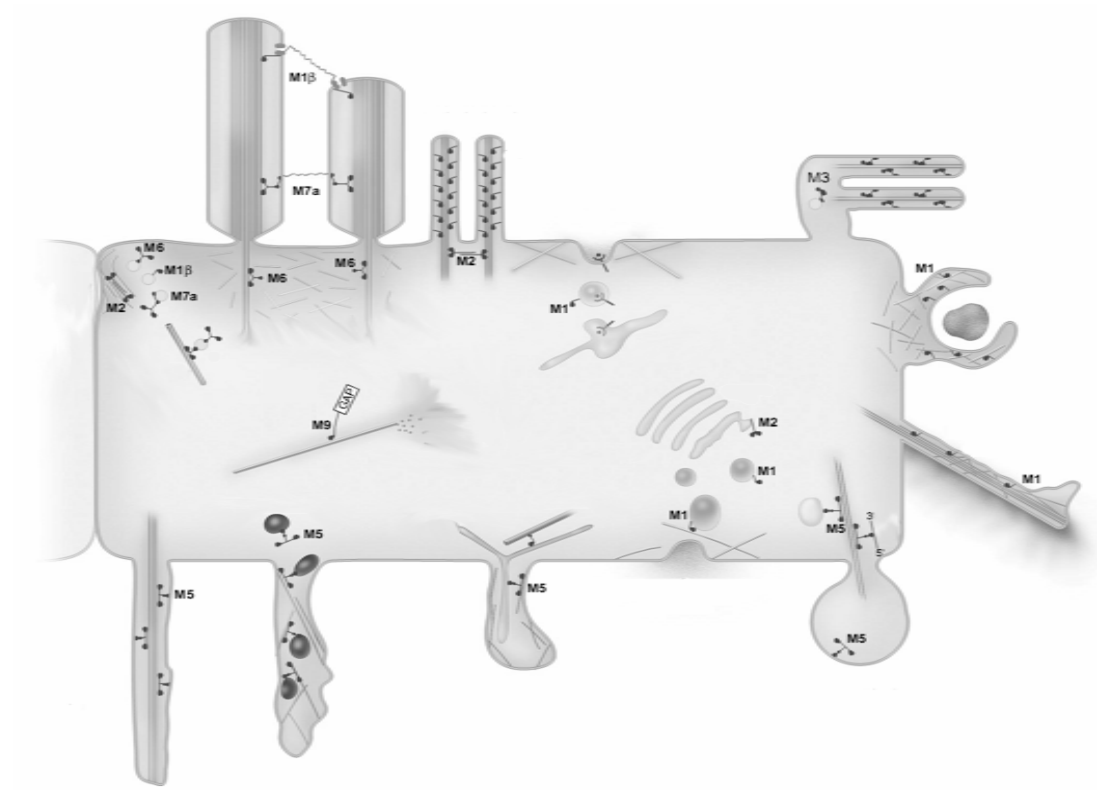

Figura 08: Funções das miosinas no citoplasma da célula. Modificado de Mermall, Post *et al.* (1998).

As miosinas também estão envolvidas no tráfego de transportadores de glicose (GLUT) (Huang e Czech, 2007). Neste sentido, a miosina-Ic (Bose, Guilherme *et al.*, 2002; Bose, Robida *et al.*, 2004; Huang, Lifshitz *et al.*, 2004; Huang, Imamura *et al.*, 2005) e a miosina-Va (Yoshizaki, Imamura *et al.*, 2007) foram encontradas mediando o transporte de vesículas de GLUT4, e a miosina-VI com o GLUT1 (Reed, Cefalu *et al.*, 2005). Além disso, Yoshizaki, Imamura *et al.*, (2007) demonstraram que a insulina estimula a fosforilação da miosina-Va aumentando a afinidade da proteína aos filamentos de actina, considerando que a inibição da sua função possa levar a um bloqueio do transporte de glicose estimulado pela insulina.

Recentemente, uma nova miosina foi descoberta em mitocôndria, sendo designada Myo19 (Quintero, Divito *et al.*, 2009), que possui aproximadamente 35% de identidade com a sequência do domínio motor das miosinas das classes V e VI de humano, estando expressa em células, tecidos e tumores de vertebrados. Além disso, foi revelado que este motor molecular participa da dinâmica mitocondrial normal, possui três motivos IQ e não é regulado pela fosforilação da sua cadeia pesada.

1.7.1 Miosina-IIB

A miosina-II é um hexâmero, considerado um modelo convencional de miosina, que foi primeiramente purificado de tecido muscular estriado (Sellers, 2000). Esse motor molecular é composto por duas cadeias pesadas, duas cadeias leves essenciais e duas cadeias leves regulatórias (Landsverk e Epstein, 2005; Conti e Adelstein, 2008) (Figura 09), podendo ser encontrado nas células dos músculos esquelético, cardíaco e liso, bem como no citoplasma das células não-musculares (Sellers, 2000).

Em vertebrados existem mais de 15 isoformas de cadeias pesadas de miosina-II, geradas por diferentes genes e por *splicing* alternativo (Conti e Adelstein, 2008). Em mamíferos, três isoformas de miosina-II não-muscular são conhecidas, IIA, IIB e IIC, podendo ser codificadas por três genes específicos, *MYH9*, *MYH10* e *MYH14*, respectivamente (Katsuragawa, Yanagisawa *et al.*,

1989; Kawamoto e Adelstein, 1991; Simons, Wang *et al.*, 1991; Bresnick, 1999; Berg, Powell *et al.*, 2001; Golomb, Ma *et al.*, 2004).

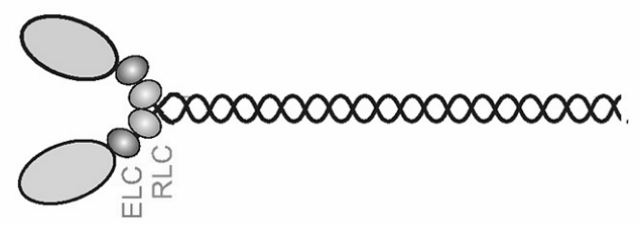

Figura 09: Esquema da molécula de miosina-II com a sua cadeia pesada e cadeias leves essencial (ELC) e regulatória (RLC). Modificado de Lowey e Trybus, 2010.

As isoformas IIA e IIB se localizam diferencialmente dentro das células e essa distribuição sugere que as duas proteínas tenham importâncias funcionais distintas (Maupin, Phillips *et al.*, 1994; Rochlin, Itoh *et al.*, 1995; Kelley, Sellers *et al.*, 1996), estando amplamente expressas em neurônios do córtex, cerebelo e cordão espinhal (Kawamoto e Adelstein, 1991; Miller, Bower *et al.*, 1992; Itoh e Adelstein, 1995).

Em células não-musculares, a miosina-II tem diversas funções, que variam desde a citocinese, migração neuronal e prolongamento de neuritos, até tráfego de membrana dentro da célula, exocitose e transporte de organelas em axônios (De Lozanne e Spudich, 1987; Knecht e Loomis, 1987; Mochida, Kobayashi *et al.*, 1994; Mochida, 1995; Wylie, Wu *et al.*, 1998; Tullio, Bridgman *et al.*, 2001; Wylie e Chantler, 2001; Degiorgis, Reese *et al.*, 2002).

As atividades funcionais das cadeias leves e pesadas da miosina-IIB são reguladas por fosforilação (Bresnick, 1999), envolvendo diferentes vias e enzimas que podem afetar outros aspectos da dinâmica do citoesqueleto, incluindo as alterações no movimento, divisão celular ou secreção (Spudich, 1994). De todas as formas de miosina, as da classe II têm sido estudadas mais extensivamente e parecem ter papel na organização e comportamento do citoesqueleto de cones de crescimento (Vallee, Seale *et al.*, 2009). Além disso, já foi descrito que a remoção total ou parcial desta miosina pode levar a danos no sistema nervoso em resultado de um defeito na migração celular (Brown e Bridgman, 2004).

Dentre as isoformas, a IIB é a mais enriquecida em cérebro (Murakami, Mehta *et al.*, 1991) e possui localização cortical no corpo celular e axônio, funcionando como um mediador da motilidade em cones de crescimento (Cheng,

Murakami *et al.*, 1992; Rochlin, Itoh *et al.*, 1995). Além disso, a cadeia leve desta miosina pode interagir com as subunidades do receptor NMDA (Husi, Ward *et al.*, 2000; Amparan, Avram *et al.*, 2005), funcionando como um importante regulador da morfologia dos dendritos neuronais (Ryu, Liu *et al.*, 2006).

1.7.2 Miosina-Va

A miosina-V foi inicialmente caracterizada como uma proteína ligante de calmodulina no cérebro, com várias propriedades bioquímicas semelhantes às miosinas (Larson, Pitta *et al.*, 1988; Larson, Espindola *et al.*, 1990; Espindola, Espreafico *et al.*, 1992; Cheney, O'shea *et al.*, 1993; Coelho e Larson, 1993; Nascimento, Cheney *et al.*, 1996)

A cadeia pesada de miosina-V consiste de três domínios (Figura 09): 1) domínio motor com duas cadeias pesadas com, aproximadamente, 212 kDa (Espreafico, Cheney *et al.*, 1992; Cheney, O'shea *et al.*, 1993) e com alta afinidade pela actina na presença de ATP (Espreafico, Cheney *et al.*, 1992; Cheney, O'shea *et al.*, 1993); 2) domínio pescoço, contendo seis sítios ligantes de cadeias leves, com 4 a 5 moléculas de calmodulina para cada cadeia pesada (Espreafico, Cheney *et al.*, 1992; Cheney, O'shea *et al.*, 1993), e duas cadeias leves essenciais de 17 kDa e 23 kDa; 3) domínio cauda dividido em duas regiões: um domínio globular C-terminal (Espreafico, Cheney *et al.*, 1992; Cheney, O'shea *et al.*, 1993) e uma região alfa-helicoidal *coiled-coil* que está envolvida na dimerização e possui uma sequência PEST (Rogers, Wells *et al.*, 1986; Espreafico, Cheney *et al.*, 1992) rica em aminoácidos prolina, ácido glutâmico, serina e treonina, considerado um importante sítio para proteólise mediada pela calpaína (Rechsteiner e Rogers, 1996). A esta região também se liga uma cadeia leve de dineína (8-10 kDa) (Benashski, Harrison *et al.*, 1997; Espindola, Suter *et al.*, 2000; Hodi, Nemeth *et al.*, 2006) que possivelmente estabiliza a interação entre as cadeias pesadas, e auxilia na ligação da carga à miosina-V (Reck-Peterson, Provance *et al.*, 2000).

Na presença de íons cálcio, a atividade ATPase da miosina-V é aumentada, permanecendo-se numa conformação mais compactada que permite

a interação do domínio cauda globular com a região cabeça-pescoço, inibindo a sua atividade mecano-enzimática (Li, Jung *et al.*, 2006).

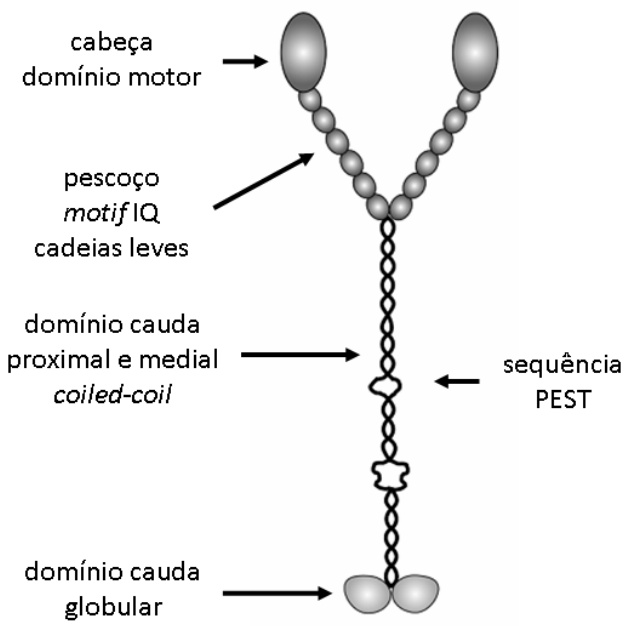

Figura 09: Estrutura dos domínios da miosina-Va. Modificado de Trybus (2008).

O domínio cauda da miosina-V se liga diretamente a várias proteínas citoplasmáticas, incluindo sinaptobrevina, sinaptofisina, sintaxina e CaMKII (Prekeris e Terrian, 1997; Evans, Lee *et al.*, 1998; Costa, Mani *et al.*, 1999; Ohyama, Komiya *et al.*, 2001), como também melanofilina (Provance, James *et al.*, 2002) e microtúbulos (Cao, Chang *et al.*, 2004). *In vitro*, a miosina-V é um substrato da CaMKII e pode atuar estimulando a atividade desta quinase no requerimento adicional de calmodulina, sugerindo que a miosina-V cerebral ativa a CaMKII doando moléculas de calmodulina (Costa, Mani *et al.*, 1999).

As funções deste motor molecular são variadas, incluindo o tráfego de membranas, organelas, RNA, melanossomos e vesículas, como também a remodelagem de membrana (Kogel, Rudolf *et al.*, 2010).

Em vertebrados são conhecidas três subclasses de miosina-V expressas de forma tecido-específico: 1) Va, altamente expressa em tecido nervoso, estando a proteína amplamente distribuída em todo o cérebro, nervo periférico e em órgãos endócrinos (Mercer, Seperack *et al.*, 1991; Espindola, Espreafico *et al.*, 1992; Evans, Hammer *et al.*, 1997; Drengk, Kajiwara *et al.*, 2000; Suter, Espindola

et al., 2000; Rose, Lejen *et al.*, 2002; Rudolf, Kogel *et al.*, 2003; Varadi, Tsuboi *et al.*, 2005; Watanabe, Nomura *et al.*, 2005; Espindola, Banzi *et al.*, 2008) 2) Vb, presente em diferentes tecidos (Zhao, Koslovsky *et al.*, 1996), incluindo o epitelial, mas com distribuição limitada no cérebro, especialmente no hipocampo, giro denteado, amígdala e córtex (Zhao, Koslovsky *et al.*, 1996; Lapierre, Kumar *et al.*, 2001; Swiatecka-Urban, Talebian *et al.*, 2007); 3) Vc, presente em tecidos exócrinos, como o pâncreas, a próstata e a glândula mamária, além do cerebelo (Bridgman e Elkin, 2000; Rodriguez e Cheney, 2002; Marchelletta, Jacobs *et al.*, 2008; Jacobs, Weigert *et al.*, 2009).

O cérebro possui grande quantidade de miosina-Va, o que sugere um envolvimento na transmissão sináptica. Dados de imunodetecção em cérebro e cerebelo de ratos mostraram intensa marcação nas extensões dendríticas das células de Purkinje e na região perinuclear (Espindola, Espreafico *et al.*, 1992; Tilelli, Martins *et al.*, 2003), sendo as vesículas e as organelas as principais cargas transportadas por esta miosina nos neurônios e em outros tipos celulares (Langford e Molyneaux, 1998; Depina e Langford, 1999,).

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Capítulo 2

Superexpressão de miosina-IIB no cérebro de ratos diabéticos induzidos por estreptozotocina

1. ARTIGO CIENTÍFICO

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OVEREXPRESSION OF MYOSIN-IIB IN BRAINS OF A STREPTOZOTOCIN-INDUCED DIABETES RAT MODEL

Luciana Karen Calábria[1], Gabriel Costa Nunes da Cruz[2], Rafael Nascimento[1], Washington João Carvalho[1], Neire Moura de Gouveia[1], Fernanda Vieira Alves[1], Fabiana Barcelos Furtado[1], Marcelo Valle de Sousa[2], Luiz Ricardo Goulart[1], Foued Salmen Espindola[1].

[1]*Institute of Genetics and Biochemistry, Federal University of Uberlândia, Campus Umuarama, 38400-902, Uberlândia-MG, Brazil.*

[2]*Brazilian Center for Protein Research, Department of Cell Biology, University of Brasília, Campus Darcy Ribeiro, 70910-900, Brasília-DF, Brazil.*

Abstract

Ca^{+2}/calmodulin complex interacts with and regulates various enzymes and target proteins, known as calmodulin-binding proteins (CaMBPs). This group of proteins includes molecular motors such as myosins. Non-muscle myosin-IIB was shown in our study to be overexpressed in diabetic rat brains. In this study, we compared CaMBPs, purified by immobilized-calmodulin, affinity chromatography, isolated from non-diabetic and streptozotocin-induced diabetic rat brains. Proteins eluted with EGTA and urea were separated on a SDS-PAGE gel, digested and submitted to peptide mass fingerprinting analysis. There were fifteen and sixteen intense bands for non-diabetic and diabetic brains, respectively. Two proteins were exclusively in non-diabetic brains, four were found exclusively in diabetic brains and thirteen were found in both. Protein sequences were then analyzed for the presence of the calmodulin-binding sites. A large fraction of the eluted proteins (83%) had putative IQ motifs or calmodulin-binding sites. The myosin-IIB affinity chromatography elution in association with western blot, immunohistochemistry and RT-PCR analyses strongly suggest that myosin-IIB protein and mRNA are highly expressed levels in diabetic rat brains. This is the first study that identified and showed calmodulin-binding proteins differential expression in non-diabetic and diabetic rat brains through a comparative proteomic analysis, and it opens up a new field of study relating myosin-IIB expression in the brain, diabetes mellitus and intracellular calcium regulation.

Key-words: Diabetes mellitus; brain; calcium; calmodulin; myosin-IIB.

1. Introduction

Diabetes mellitus is a common metabolic disorder characterized by hyperglycemia that affects the central nervous system, causing alterations in neurotransmission, electrophysiological abnormalities, structural changes and moderate disturbances in learning and memory [1-5]. Hyperglycemia causes an acute rise in cytosolic calcium concentrations due to increased calcium influx into cells, and in certain cells, hyperglycemia causes the release of intracellular calcium stores as well. Additionally, hyperglycemia has been associated with decreased calcium efflux [6]. The combination of increased calcium influx and decreased calcium efflux leads to sustained elevation of the basal levels of cytosolic calcium, which may adversely affect cell functions. Poorly controlled diabetes mellitus has been shown to increase basal cell death in specific areas of the brain [7]; thus, this could alter the levels and functions of Ca^{+2}/calmodulin-binding proteins.

Based on calcium ion dependence for calmodulin binding, it is possible to classify calmodulin-binding proteins into three categories: Ca^{+2}-dependent, Ca^{+2}-independent and Ca^{+2}-inhibited [8]. Myosins constitute a large family of actin-based motor proteins [9], for instance myosin-II. Most non-muscle cells express myosin-IIA, myosin-IIB, and myosin-IIC motor protein isoforms. Each myosin-II motor protein exists as a complex consisting of two copies each of heavy chain, essential and regulatory light chains, whose functional activities are regulated by phosphorylation [10]. The myosin II isoforms showed differences in their biological properties, tissue distribution and intracellular localization, indicating that each isoform might perform different cellular functions [10, 11]. Previous report has shown that total or partial ablation of myosin-IIB can lead to damage to the nervous system as a result of a defect in migration [12].

In the present investigation, we identified calmodulin-binding proteins in non-diabetic and diabetic rat brains and showed that non-muscle myosin heavy chain IIB is overexpressed in diabetic rat brains.

2. Material and methods

2.1. Animals

Male Wistar rats (weight between 180–220g) were housed under standard conditions (22±1°C, humidity 60±5%, 12 h light/12 h dark cycle) with food and water *ad libitum*. All procedures for the handling, use and euthanasia of animals followed the resolutions proposed by the Brazilian Society of Science in Laboratory Animals and by the Ethics Committee in Animal Research of the Federal University of Uberlândia, Brazil.

2.2. Induction of Diabetes mellitus

Rats were starved for 24 h, anesthetized by intraperitoneal injection of xylazine/ketamine (1:1 v/v), and then injected with streptozotocin (40 mg/kg body weight, 0.01 M citrate buffer, pH 4.5; Sigma-Aldrich) into the penile vein (2 mL/kg). Fasting continued 90 min after injection. Ten days after injection of streptozotocin, rats with fasting blood glucose levels above 200 mg/dL were scored diabetic. The glycemia was monitored for the subsequent 10 days with Biocheck Glucose Test Strips (Bioeasy). Animals (n = 32) were sacrificed by decapitation 20 days after the induction of diabetes and brains from diabetic and non-diabetic rats were surgically removed.

2.3. Affinity chromatography

Calmodulin-coupled Sepharose-4B resin affinity chromatography (CaM-sepharose-4B, Amersham Pharmacia Biotech) was carried out as previously described [13]. The resin was loaded into a glass column (10 x 0.5 cm) and equilibrated with 10 volumes of equilibration buffer (50 mM Tris-HCl pH 7.5, 2 mM $CaCl_2$, 1 mM β-mercaptoethanol) containing 100 mM NaCl. Four diabetic and four non-diabetic brains were homogenized on ice in three volumes of homogenization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethane sulfonyl-fluoride, 0.1 mM aprotinin, 20

ug/mL leupeptin, 0.1 mM pefabloc). The homogenate was centrifuged at 15,000 x *g* for 30 min at 4°C. Supernatants were prepared for CaM-Sepharose-4B affinity chromatography by adding 2 mM $CaCl_2$ and were applied to the column. The excluded volume was discarded, and the resin was washed with 10 volumes of equilibration buffer containing 200 mM NaCl and followed by 10 volumes of the same buffer containing 500 mM NaCl. CaMBPs were eluted with buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 1 mM β-mercaptoethanol, 100 mM NaCl and 0.2 mM PMSF, followed by elution with buffer containing 50 mM Tris-HCl (pH 7.5), 6 M urea, 1 mM β-mercaptoethanol and 0.2 mM PMSF. Affinity chromatography was performed without specific protection of phosphate/phosphatases and purification was carried out in triplicate. Protein content in the loaded supernatants and eluted fractions was estimated following a modification of Bradford assay [14] and samples with protein concentrations below 2 μg/μL were treated with 10% trichloracetic acid for 15 min on ice, and centrifugated at 12,000 x *g* for 10 min at 4°C. The protein precipitate was then solubilized in a small volume of electrophoresis sample buffer containing an additional 100 mM Tris-HCl (pH 8.0) and 25% glycerol, analyzed on a 5-22% gradient SDS-PAGE [15], and stained with Coomassie Brilliant Blue R-250.

2.4. Protein digestion and mass spectrometry

Calmodulin-binding proteins present the eluted fractions were destained in the polyacrylamide gel. Tryptic digests were prepared on an AnchorChip^{TM} plate (Bruker Daltonics) according to the modified method of Zhang et al. [16]. Mass spectra were obtained using an Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in delayed extraction and reflector modes. The spectra were processed using the FlexAnalysis 2.4 and BioTools 3.0 software tools (Bruker Daltonics). Peptide masses (MH^+) were recorded in the range of 700-3500 Da. Internal calibration was performed using known trypsin autolysis ion peaks (842.50 and 2211.09).

2.5. Database search

Monoisotopic masses of tryptic peptides were used to identify the proteins by peptide mass fingerprinting. Proteins searches in the NCBI (National Center for Biotechnology Information) database were carried out using Mascot software [17], assuming a *p*-value < 0.05. Error tolerance for peptide mass was lower than 100 ppm, and no restrictions were imposed on protein molecular mass or phylogenetic lineage. Searches that provided no significant scores were then restricted to Rodentia. Further search parameters were the presence of one missed cleavage and the presence of sites for modifications, including methionine oxidation, cysteine carbamidomethylation and N-terminal acetylation (protein). Identified proteins were categorized via UniProt/TrEMBL. Those proteins that could not be categorized using this method were classified according to cellular localization using the Psort II database (http://psort.nibb.ac.jp/form2.html). Considering the primary sequence of the identified proteins, a web-based database (http://calcium.uhnes.utoronto.ca/ctdb) was used for identification of putative CaM-binding motifs, which were classified as “potential IQ motif,” “potential motif 1-5-10 subclass,” “potential CaM-binding site,” “nonspecific CaM-binding site” and “absent CaM-binding motif” [18, 19].

2.6. Western Blotting

Myosin-IIB expression was evaluated in diabetic and non-diabetic brain homogenates (*n* = 3, respectively) in homogenization buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM EGTA, 5 mM ATP, 2 mM dithiothreitol, 1 mM benzamidine, 0.5 mM phenylmethane sulfonyl-fluoride, 0.1 M aprotinin, 20 ug/mL leupeptin, 0.1 mM pefabloc). The homogenates were cleared by centrifugation at 15,000 x *g* for 30 min at 4ºC. Protein content in the supernatants was estimated using the Bradford assay and solubilized in a small volume of electrophoresis sample buffer containing an additional 100 mM Tris-HCl, pH 8.0, and 25% glycerol. All supernatant samples containing 30 μg of protein were analyzed on a 5-22% SDS-PAGE and electroblotted on nitrocellulose membranes in Tris-glycine buffer [20]. Membranes were then incubated with 5% dried milk in TBS-T (50 mM

Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and then probed with anti-myosin-IIB primary antibody diluted to 0.2 µg/mL. Following washes with TBS-T, blots were incubated with a peroxidase-conjugated anti-rabbit IgG (GE Healthcare; diluted 1:2000) and washed several times with TBS-T. Antibodies bound to the membranes were visualized by chemiluminescence after treatment with ECL™ (GE Healthcare) followed by exposure to Hyperfilm™ (GE Healthcare) following manufacturer's instructions. The intensity of the protein bands was analyzed and compared using Scion Image software, version Alpha 4.0.3.2 (Scion Corporation) and results were expressed as percentage of total content.

2.7. RNA isolation and cDNA synthesis

Total RNA was isolated from four diabetic and four non-diabetic brains using TRIzol reagent (Invitrogen) following manufacturer's instructions and then resuspended in DEPC-treated water. Its quality and quantity was established by reading the optical density of each sample at 260 and 280 nm using NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies) and agarose gel electrophoresis. One microgram of total RNA was reverse transcribed at 37°C for 1 h in a 20 µL reaction mixture containing the first strand buffer, 40 U Murine Monoley Leukemia Virus Reverse Transcriptase (Invitrogen), 0.25 mM of each dNTP, 10 U RNAsin (Invitrogen), 0.5 mM DTT, and 126 pmol hexamer random primers (Invitrogen).

2.8. Preparation of quantification standard

Standards were prepared by cloning PCR products of *MYH10* and the housekeeping beta-2-microglobulin (*B2M*) fragments using TOPO TA Cloning Dual Promoter Kit (Invitrogen). The recombinant plasmid DNA was isolated and sequenced using MegaBACE 1000 automatic sequencer (Molecular Dynamics). The sequencing reaction was carried out using the DyEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare) following manufacturer's instructions. Plasmid concentration was measured and the copy numbers calculated according to Yin et al [21]. Serial dilutions of each standard were made in the range 10 to 10^7

copies per µL for *MYH10* and *B2M*. The efficiency reaction was calculated according to a formula, $E = (10^{-1/slope} - 1) \times 100$, where the log of the each dilution was plotted with ΔC_T of housekeeping and target genes.

2.9. Real-time PCR conditions

The qPCR assay was carried out in 7300 Real-time PCR System (PE Applied Biosystems) using SYBR Green qPCR Master Mix reagent (Fermentas). The thermal cycling profile used was the Universal Program (PE Applied Biosystems). Primers were designed between exons junctions to avoid amplification of contaminating genomic DNA using Primers Express software (PE Applied Biosystems). For *B2M* fragment amplification, the set of primers were: 5'-CGT CGT GCT TGC CAT TCA-3' and 5'-TCC TCA ACT GCT ACG TGT CTC AG-3'. The *MYH10* forward and reverse primers were respectively: 5'-CCA TGC CGG AGA ACA CAG T-3' and 5'-AAG CCC AGA CCA AAG AGC AG-3'. The relative expression of each specific product was calculated by $2^{-\Delta\Delta CT}$ (C_T = fluorescence threshold value; $\Delta C_T = C_T$ of the target gene - C_T of the reference gene (*B2M*); $\Delta\Delta C_T = \Delta C_T$ of the target sample - ΔC_T of the calibrator sample). All samples were run in duplicates.

2.10. Immunohistochemistry

Brains were dissected and fixed with 10% formaldehyde solution in phosphate-buffered saline 0.1 M (pH 7.4) for 24 h, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Five micrometer sections were pretreated with 4 mM citrate buffer (pH 6.0), containing 0.025% Tween 20, in a microwave for 5 min. Thereafter, sections were incubated with anti-myosin-IIB for 16 h followed by incubation with the Post Primary Block, NovoLink™ Max Polymer Detection System (Novocastra Laboratories Ltd). After three washes with TBS-T, the sections were incubated with the NovoLink polymer for 30 min at 37°C. Chromogen development was performed with 3,3'-diaminobenzidine, and the material was counterstained with Harris hematoxylin, dehydrated, mounted with

Permount and analyzed using a light microscope (Zeiss Axiolab). For negative controls, the primary antibody was omitted in the reaction.

2.11. Statistical analysis

Statistical analysis was carried out by the Student t-test. The data were analyzed using the SigmaStat 3.5 software (Systat Software Inc.). Means and standard deviations were calculated. A p-value < 0.05 was considered significant.

3. Results and Discussion

In this study, we identified proteins of non-diabetic and diabetic rat brains that interact with the Ca^{+2}/calmodulin complex. SDS-PAGE analysis of the EGTA-eluted fractions from a CaM-Sepharose-4B column revealed a similar protein profile for both types of brains, with fifteen intense bands for non-diabetic and sixteen for diabetic brains (Figure 1). The fraction eluted with 6 M urea revealed two highly expressed proteins in diabetic brains: myosin-IIB and cytoplasmic actin. Two calmodulin-binding proteins were found only in non-diabetic brains, four were exclusively identified in diabetic and thirteen were found in both types of rat brains, as indicated in Table 1. The sequences of all identified proteins were verified in the database with respect to the presence of a calmodulin-binding motif. The proteins were classified as “potential IQ motif” (two), “potential motif 1-5-10 subclass” (two), “potential calmodulin-binding site” (eleven), “nonspecific calmodulin-binding motif” (two) and “absent calmodulin-binding motif” (one), as shown in Table 1.

Two metabolic enzymes were identified, phosphofructokinase and acyl CoA synthetase, that are well-known calmodulin-binding proteins. Although, phosphofructokinase have been identified in both brains, there is report that the levels of this enzyme are decreased in diabetic brains [22]. Cytoplasmic proteins were also identified in the non-diabetic and diabetic brains, including glycolipid transfer protein, a protein that accelerates the intermembrane transfer of various glycolipids [23]; ubiquitin protein ligase E3C, which is thought to be the component of the ubiquitin conjugation system most directly responsible for substrate recognition [24, 25] and modulating the function of the synapse [26]; and heat

shock protein 8 (HSP70). Studies have revealed that calmodulin binds to HSP70 in a calcium-dependent manner [27], and activates calcineurin via a calmodulin-dependent and independent pathways [28]. It is known that members of the HSP70 family can act as chaperones, regulating the folding and stability of several proteins, including receptors and signal transduction regulators, or as biomarkers of cellular death [29].

Calmodulin is present in the nucleus of different cell types, suggesting that it is involved in the regulation of nuclear functions [8]. Three proteins predicted by Psort II to be localized to the nucleus were also detected, such as zinc finger protein 260, similar centromere protein F and microtubule-actin crosslinking factor 1. The identification of nuclear proteins was not unexpected, as subcellular fractionation of biological samples is seldom complete [30]. Furthermore, all of these proteins interact with calmodulin and contain calmodulin-binding motifs.

Both the alpha and beta isoforms of Ca^{+2}/calmodulin-dependent protein kinase II (CaMKII) were identified. These abundant neural proteins mediate diverse physiological responses to increases in intracellular Ca^{+2} concentrations by Ca^{+2}/calmodulin complex activation in neurons [31]. The alpha and beta isoforms are neuron specifics, and highly abundant in rat brains [32]. CaMKII seems to be relatively vulnerable to pathological states, which are associated with massive Ca^{+2} influxes into cells [33]. Activity and enzyme content of CaMKII are increased in diabetic rat brains, and this change may be a molecular cause of previously reported deficits in learning and hippocampal plasticity [33, 34]. This enzyme was identified in four distinct bands in non-diabetic and diabetic brains that differed slightly in mass, suggesting that the CaMKII proteins may be post-translationally modified, including phosphorylation, which is a key factor in the trafficking and functioning of this protein [35].

Many of the identified proteins, including actin, dynein, myosin, spectrin and tubulin, are implicated in cytoskeleton function. Modification of actin expression pattern in diabetic brains may be related to structural changes driven by the reorganization of actin-microfilaments in the cell during the evolution of diabetes. Changes in neuronal activity can bring about rapid alteration of the size and morphology of these structures [36, 37] which in turn may change the synaptic efficacy, in particular the dynamics of actin [38]. In another proteomic analysis of

postsynaptic density, many actin-regulatory proteins were identified, as well as an insulin receptor substrate protein that, when associated, increases the dynamics of actin [39].

For the first time, we report here myosin-IIB overexpression in diabetic brains. Although, myosin-IIB lacks a calmodulin binding site, this molecular motor has a regulatory light chain that interacts and it is regulated by a myosin light chain kinase that has calmodulin-binding site. Thus, myosin-IIB may have been co-purified with others calmodulin-binding molecules in our experiment.

Analysis revealed 61 peptides with *m/z* ratios ranging from 704.3800 to 2532.1070, in accordance with the predicted mass of *in silico*-digested non-muscle myosin heavy chain IIB (Figure 2A). The myosin-IIB identified in this search has the accession number 13928704, an estimated molecular mass of 229.79 kDa and an isoelectric point of 5.49 (Figure 2B). Searches considering these peptides masses using Mascot in the NCBI protein database covered 34% of the *Rattus norvegicus* myosin-IIB sequence (Figure 2C).

To evaluate expressed protein concentration of myosin-IIB, western blot analysis was done using supernatant from rat brains and exhibited an increase of about 59% in brain of diabetic rats compared with non-diabetic rats ($p < 0.05$) (Figure 3). The differential expression of myosin-IIB protein in non-diabetic and diabetic brains, as found by affinity chromatography, SDS-PAGE and western blotting, was validated considering the *MYH10* mRNA expression by real-time PCR, and distribution of myosin-IIB protein by immunohistochemistry.

Real-time PCR (Figure 4) revealed that *MYH10* mRNA was significantly 1.4-fold higher in diabetic brain ($p < 0.001$) than non-diabetic, in agreement with western blot. Amplification efficiency was tested by standard curves for *Rattus novergicus B2M* ($R^2 = 0.9997$) and *MYH10* ($R^2 = 0.996$) generated by plotting the value of C_T cycle *vs*. the log of plasmid concentration (from 10^4 to 10^6 copies). Linear regression analysis was used to determine the slope which corresponds to the amplification efficiency. Slope value of about -3.2 and $R^2 \geq 0.99$ were admitted to reaction efficiency of 100% (Figure 4B).

As shown in Figure 5, myosin-IIB protein distribution was analyzed by immunohistochemistry. In the cerebral cortex, myosin-IIB immunoreactivity was observed mainly in pyramidal neurons that are known to be projecting neurons.

Neuronal and glial cells of the frontal and temporal cortex exhibited increase immunostaining for myosin-IIB in diabetic rat brains. Nevertheless, myosin-IIB distribution on others regions of the brain is the same for non-diabetic and diabetic rats. In normal rat brain, the distribution of nonmuscle myosin isoforms (myosin-IIA and myosin-IIB) was previously reported and our results are in according with it [40].

Disturbance in insulin signaling appears to be the main common impairment that affects cell growth and differentiation, cellular repairs mechanisms, energy metabolism, and glucose levels [41]. There are data reported that IGF-I increase type IIb myosin heavy chain expression mRNA levels in skeletal muscle [42].

Extensive evidences confirmed that myosin-IIB plays a role in the secretory processes of a variety of cells, including mast cells [43], natural killer cells [44], hippocampal cells [45], sensory neurons [46], chromaffin cells [47], beta cells [48], exocrine cells [49-51] and oocytes [52]. Moreover, this myosin is abundant in neuronal cell bodies and neurites, and it is important for growth cone motility and axon outgrowth [53, 54].

On the other hand, myosin-IIA was recently localized in mitochondria [55], which could probably have relevant role during hyperglycemia. Also, it is possible that myosin-IIB expression alters due to decreased insulin levels in consequence of streptozotocin-induced diabetes, and if myosin-IIB overexpression is specific to the model, the inhibition should at least partly restore myosin levels in diabetes [56].

4. Conclusions

The identification of calmodulin-binding proteins in the brain that are expressed at different levels under distinct conditions can elucidate many important biochemical pathways and aid to discovering novel calmodulin targets. Our study is the first that describes calmodulin-binding proteins in the non-diabetic and diabetic rat brains through a comparative proteomic study, as well as the overexpression of non-muscle myosin heavy chain IIB in diabetic rats. This report opens up new areas of study related to the link between myosin-IIB levels in the

brain and Diabetes mellitus, a metabolic disorder that is associated with a wide variety of long term complications.

Acknowledgements

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Figures and Legends

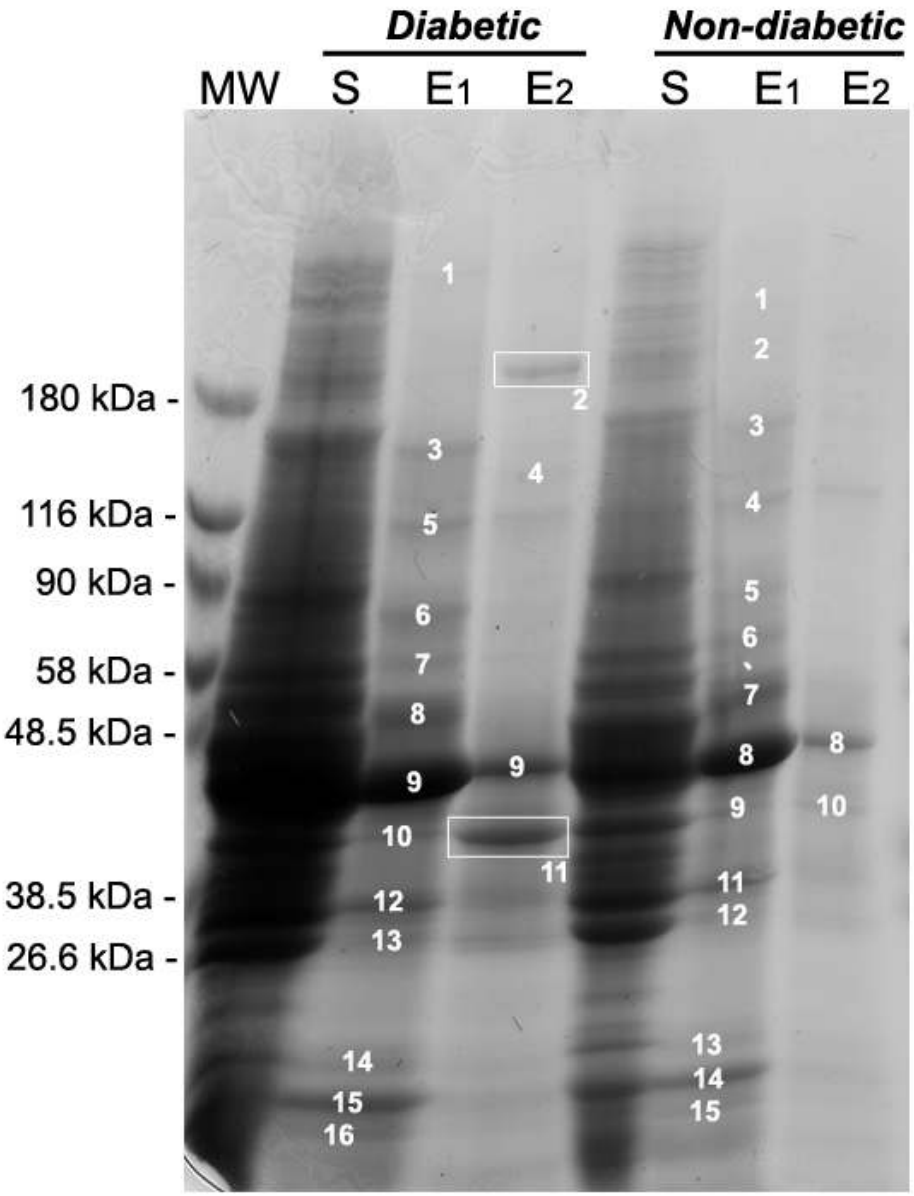
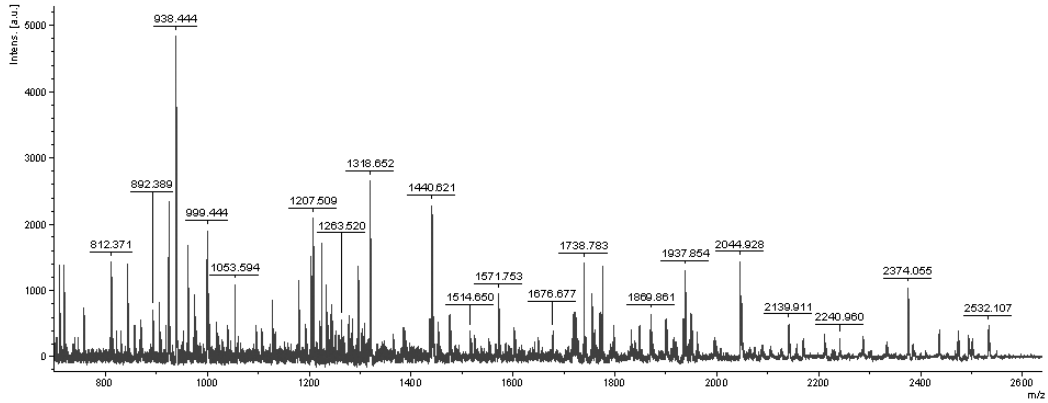


Figure 1 – Profile of calmodulin-binding proteins from brains of diabetic and non-diabetic rats. (**S**) brain supernatant; (**E_1**) proteins eluted from the CaM-Sepharose-4B column with 2 mM EGTA; (**E_2**) proteins eluted from the CaM-Sepharose-4B column with 6 M urea; (**1-16**) CaMBP bands from the brains of diabetic and non-diabetic rats excised for PMF analysis; (**Rectangles**) CaMBP band identified as non-muscle myosin heavy chain IIB (2) and cytoplasmic actin (11).

A



B

Accession number	Sequence name	Theoretical Mr/pI	Score	Expect	Sequence Coverage	Matched peptides
13928704	nonmuscle myosin heavy chain IIB [Rattus norvegicus]	229793/5.49	86	0.00019	34%	61

C

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   1 MAQRTGLEDP ERYLFVDRAV IYNPATQADW TAKKLVWIPS ERHGFEAASI
  51 KEERGDEVMV ELAENGKKAM VNKDDIQKMN PPKFSKVEDM AELTCLNEAS
 101 VLHNLKDRYY SGLIYTYSGL FCVVINPYKN LPIYSENIIE MYRGKKRHEK
 151 PPHIYAISES AYRCMLQDRK DQSILCTGES GAGKTENTKK VIQYLAHVAS
 201 SHKGRKDHNI PGELERQLLQ ANPILESFGN AKTVKNDNSS RFGKFIRINF
 251 DVTGYIVGAN IETYLLEKSR AVRQAKDERT FHIFYQLLSG AGEHLKSDLL
 301 LEGFNNYRFL SNGYIPIPGQ QDKDNFQETM EAMHIMGFSH EEILSMLKVV
 351 SSVLQFGNIS FKKERNTDQA SMPENTVAQK LCHLLGMNVM EFTRAILTPR
 401 IKVGRDYVQK AQTKEQADFA VEALAKATYE RLFRWLVHRI NKALDRTKRQ
 451 GTSFIGILDI AGFEIFELNS FEQLCINYTN EKLQQLFNHT MFILEQEEYQ
 501 REGIEWNFID FGLDLQPCID LIERPANPPG VLALLDEECW FPKATDKTFV
 551 EKLVQEQGSH SKFQKPRQLK DKADFCIIHY AGKVDYKADE WLMKNMDPLN
 601 DNVATLLHQS SDRFVAELWK DVDRIVGLDQ VTGMTETAFG SAYKTKKGMF
 651 RNVGQLYKES LTKLMATLRN TNPNFVRCII PNHEKRAGKL DPHLVLDQLR
 701 CNGVLEGIRI CRQGFPNRIV FQEFRQRYEI LTPNAIPKGF MDGKQACERM
 751 IRALELDPNL YRIGQSKIFF RAGVLAHLEE ERDLKITDII IFFQAVCRGY
 801 LARKAFAKKQ QQLSALKVLQ RNCAAYLKLR HWQWWRVFTK VKPLLQVTRQ
 851 EEELQAKDEE LLKVKEKQTK VEGELEEMER KHQQLLEEKN ILAEQLQAET
 901 ELFAEAEEMR ARLAAKKQEL EEILHDLESR VEGEEERNQI LQNEKKKMQA
 951 HIQDLEEQLD EEEGARQKLQ LEKVTAEAKI KKMEEEVLLL EDQNSKFIKE
1001 KKLMEDRIAE CSSQLAEEEE KAKNLAKIRN KQEVMISDLE ERLKKEEKTR
1051 QELEKAKRKL DGETTDLQDQ IAELQAQVDE LKVQLTKKEE ELQGALARGD
1101 DETLHKNNAL KVARELQAQI AELQEDFESE KASRNKAEKQ KRDLSEELEA
1151 LKTELEDTLD TTAAQQELRT KREQEVAELK KALEDETKNH EAQIQDMRQR
1201 HATALEELSE QLEQAKRFKA NLEKNKQGLE TDNKELACEV KVLQQVKAES
1251 EHKRKKLDAQ VQELHAKVSE GDRLRVELAE KANKLQNELD NVSTLLEEAE
1301 KKGMKFAKDA AGLESQLQDT QELLQEETRQ KLNLSSRIRQ LEEEKNSLQE
1351 QQEEEEEARK NLEKQVLALQ SQLADTKKKV DDDLGTIEGL EEAKKKLLKD
1401 VEALSQRLEE KVLAYDKLEK TKNRLQQELD DLTVDLDHQR QIVSNLEKKQ
1451 KKFDQLLAEE KGISARYAEE RDRAEAEARE KETKALSLAR ALEEALEAKE
1501 EFERQNKQLR ADMEDLMSSK DDVGKNVHEL EKSKRALEQQ VEEMRTQLEE
1551 LEDELQATED AKLRLEVNMQ AMKAQFERDL QTRDEQNEEK KRLLLKQVRE
1601 LEAELEDERK QRALAVASKK KMEIDLKDLE AQIEAANKAR DEVIKQLRKL
1651 QAQMKDYQRE LEEARASRDE IFAQSKESEK KLKSLEAEIL QLQEELASSE
1701 RARRHAEQER DELADEIANS ASGKSALLDE KRRLEARIAQ LEEELEEEQS
1751 NMELLNDRFR KTTLQVDTLN TELAAERSAA QKSDNARQQL ERQNKELKAK
1801 LQELEGAVKS KFKATISALE AKIGQLEEQL EQEAKERAAA NKLVRRTEKK
1851 LKEIFMQVED ERRHADQYKE QMEKANARMK QLKRQLEEAE EEATRANASR
1901 RKLQRELDDA TEANEGLSRE VSTLKNRLRR GGPISFSSSR SGRRQLHIEG
1951 ASLELSDDDT ESKTSDVNET QPPQSE
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Figure 2 – MALDI-TOF MS spectrum and properties of myosin-IIB. (**A**) Spectrum of the peptide masses generated from the digested band showing the *m/z* (mass to charge ratio) (*x*-axis) and the intensity of the molecular ions (*y*-axis). From the results of the MS analysis, all peaks represent peptides used for identification of the protein as myosin-IIB by PMF. (**B**) Table of the main characteristics of the myosin-IIB identification in the database. (**C**) Myosin-IIB sequence (accession number 13928704) indicating the regions covered by the 61 peptides from the spectrum (underlined/bold).

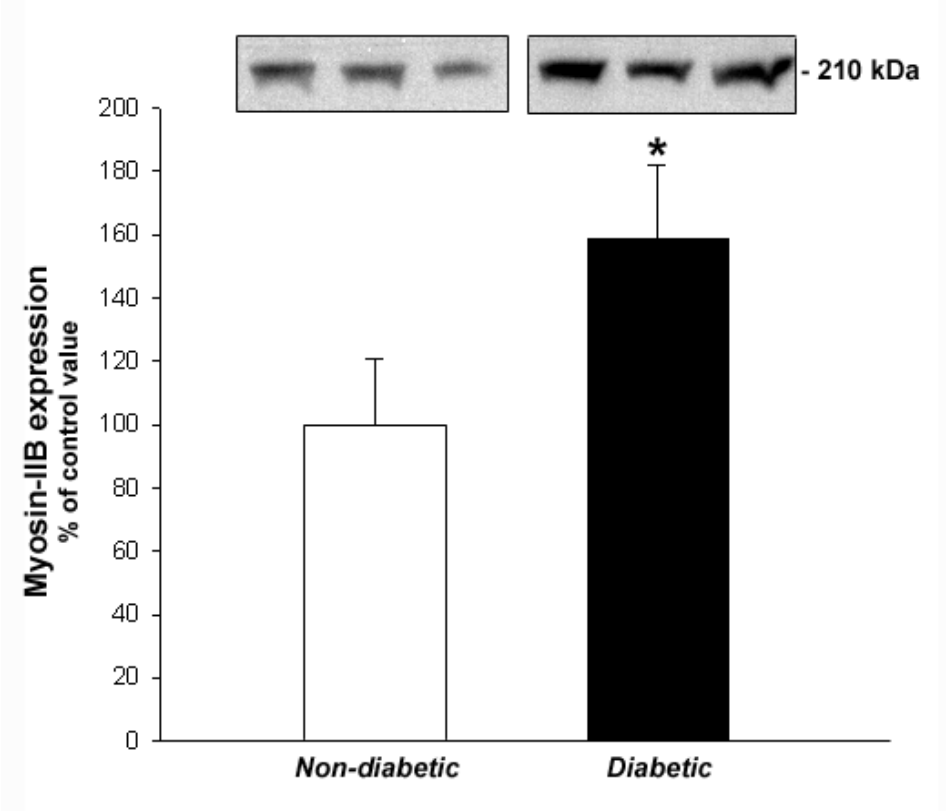

Figure 3 – Myosin-IIB expression in non-diabetic and diabetic rat brains. Western blots and quantification of myosin-IIB expression from supernatant samples of non-diabetic and diabetic rat brains. The amount of myosin-IIB protein presented on the immunoblot was determined densitometrically and expressed as a related percentage of the non-diabetic and diabetic groups. (*) $p < 0.05$, $n = 3$ rats/group.

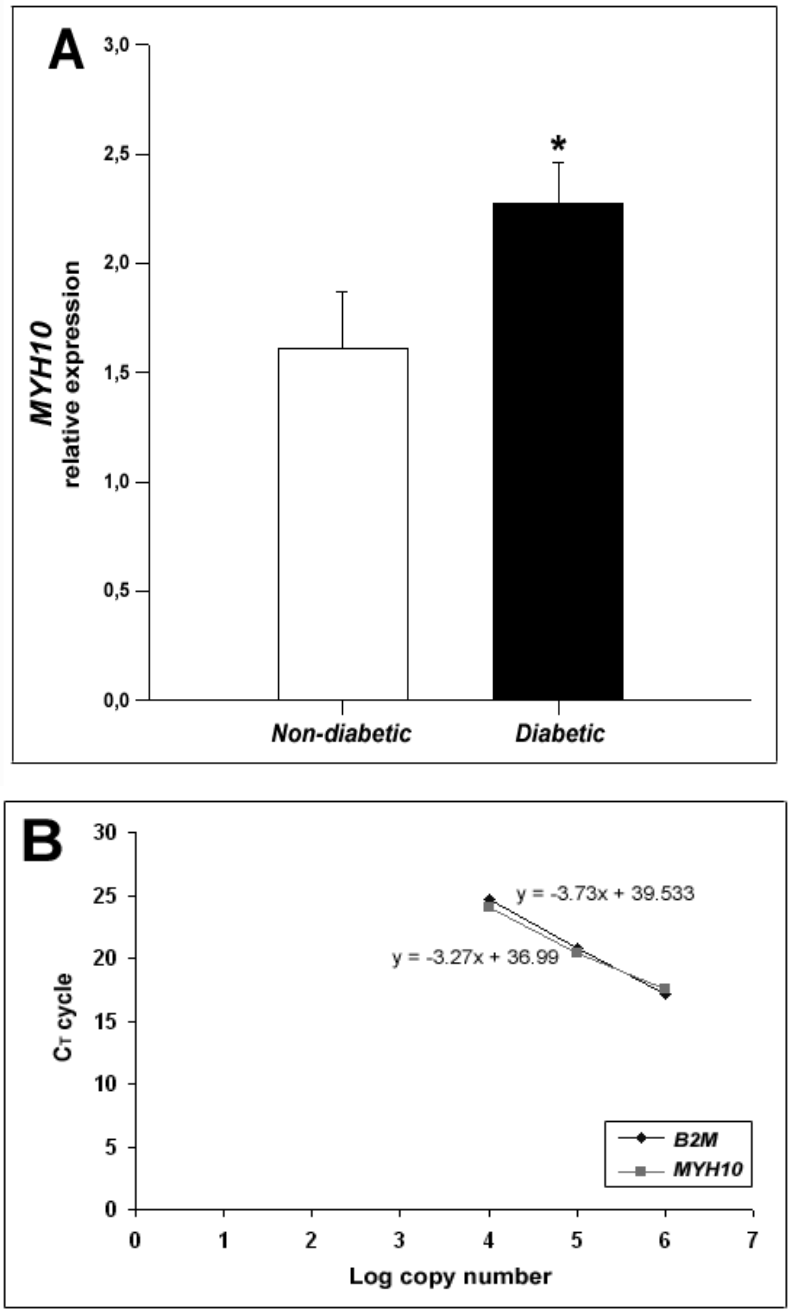

Figure 4 – *MYH10* expression in the non-diabetic and diabetic rat brains. (**A**) The box-plot diagram shows significantly upregulated *MYH10* mRNA expression in the diabetic compared non-diabetic brain samples. (*) $p < 0.001$, $n = 6$ rats/group. (**B**) Standard curves for *Rattus novergicus B2M* ($R^2 = 0.9997$, slope = -3.73) and *MYH10* ($R^2 = 0.996$, slope = -3.27) generated by plotting the value of C_T cycle vs. the log of plasmid concentration (from 10^4 to 10^6 copies). Linear regression graphic was used to determine the amplification efficiency.

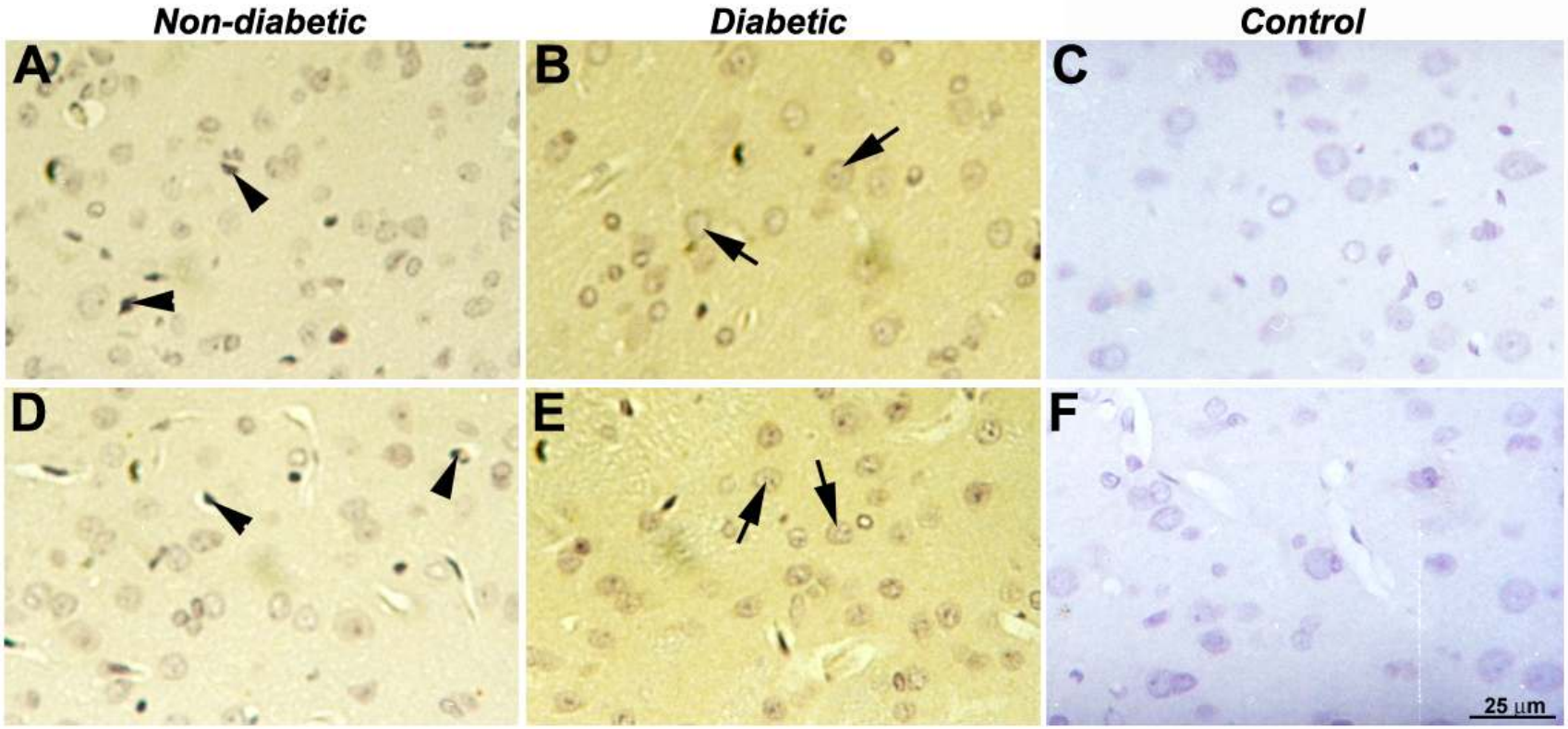

Figure 5 – Comparison of myosin-IIB distribution in non-diabetic and diabetic rat brain. In neuronal (**arrow**) and glial (**arrowhead**) cells of the frontal and temporal cortex, diabetic (**B, E**) exhibits higher expression for myosin-IIB than non-diabetic (**A, D**), it presented immunostaining in brown. Control (**C, F**). Bar: 25 μm.

Table 1 – Identification of calmodulin-binding proteins in the brains of diabetic and non-diabetic rats. (**D**) diabetic; (**ND**) non-diabetic; (*) CaMBPs eluted from the CaM-Sepharose-4B with column 6 M urea; (**MMt**) theoretical molecular mass (in kDa) as assigned in the NCBInr database; (**MMe**) experimental molecular mass (in kDa) based on the commercial molecular weight standards; (**PIQM**) potential IQ motif; (**1-5-10 motif**) potential motif of the 1-5-10 subclass; (**PCaMS**) potential calmodulin-binding site; (**UcM**) nonspecific calmodulin-binding site; (**NoM**) calmodulin-binding motif absent.

	Accession	Protein name	MMt	MMe	score	motif
D1	gi|148491097	Cytoplasmic dynein 1 heavy chain 1	534.45	>180	160	UcM at 3155 aa
D2*	gi|13928704	Nonmuscle myosin heavy chain IIB	229.79	>180	86	PCaMS
D3	gi|31543764	Alpha-spectrin 2	285.22	180-116	86	PCaMS
D4	gi|6978593	Calcium/calmodulin-dependent protein kinase II alpha	54.65	180-116	121	1-5-10 motif at 296
D5	gi|62646604	Similar to ubiquitin protein ligase E3C	124.86	116	183	PIQM at 48aa
	gi|57977273	Phosphofructokinase, platelet	86.63	90-58	176	PCaMS
D6	gi|149041705	Acyl-CoA synthetase bubblegum family member 1	69.77		115	PCaMS
D7	gi|13242237	Heat shock protein 8	71.05	58	61	UcM at 257 aa
D8	gi|125287	Calcium/calmodulin-dependent protein kinase II beta	61.10	48.5	220	1-5-10 motif at 297
D8*	gi|149064377	Calcium/calmodulin-dependent protein kinase II alpha	54.84	48.5-36.5	64	1-5-10 motif at 298
D9	gi|6978593	Calcium/calmodulin-dependent protein kinase II alpha	54.65		139	1-5-10 motif at 296
D10*	gi|7106439	Tubulin, beta 5	50.09	48.5-36.5	75	PCaMS
D11*	gi|109492380	Similar to actin, cytoplasmic 2 gamma	59.16	48.5-36.5	84	NoM
D12	gi|224839	Tubulin T beta15	50.36	36.5	83	PCaMS
D13	gi|8394006	Zinc finger protein 260	48.09	26.6	70	PCaMS
D14	gi|223556	Tubulin alpha	50.89	>26.6	99	PCaMS
D15	gi|197313643	Glycolipid transfer protein	23.86	>26.6	62	PCaMS
D16	gi|148667971	Tubulin, alpha 4	28.13	>26.6	72	PCaMS
ND1	gi|109499266	Similar to centromere protein F	358.01	>180	72	PCaMS
ND2	gi|209364564	Microtubule-actin crosslinking factor 1	623.21	180	62	PIQM at 2136aa and 2549aa
ND3	gi|6978593	Calcium/calmodulin-dependent protein kinase II alpha	546.51	180-116	141	1-5-10 motif at 296
ND4	gi|62646604	Similar to ubiquitin protein ligase E3C	124.86	116	213	PIQM at 48aa
ND5	gi|57977273	Phosphofructokinase, platelet	86.63	90-58	103	PCaMS
	gi|149041705	Acyl-CoA synthetase bubblegum family member 1	69.77	90-58	155	PCaMS
ND6	gi|13242237	Heat shock protein 8	71.05	58	79	UcM at 257 aa
ND7	gi|125287	Calcium/calmodulin-dependent protein kinase II beta	61.10	48.5	130	1-5-10 motif at 297
ND8	gi|6978593	Calcium/calmodulin-dependent protein kinase II alpha	54.65	48.5-36.5	100	1-5-10 motif at 296
ND8*	gi|149064377	Calcium/calmodulin-dependent protein kinase II alpha	54.84	48.5-36.5	115	1-5-10 motif at 298
ND9	gi|7106439	Tubulin, beta 5	50.09	48.5-36.5	64	PCaMS
ND10*	gi|109492380	Similar to actin, cytoplasmic 2 gamma	59.16	48.5-36.5	64	NoM
ND11	gi|224839	Tubulin T beta15	50.36	36.5	67	PCaMS
ND12	gi|148667971	Tubulin, alpha 4	28.13	26.6	63	PCaMS
ND13	gi|223556	Tubulin alpha	50.89	>26.6	103	PCaMS
ND14	gi|6959684	Glycolipid transfer protein	23.87	>26.6	85	PCaMS
ND15	gi|148667971	Tubulin, alpha 4	28.13	>26.6	117	PCaMS

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2. CONCLUSÕES

- Os cérebros dos ratos foram avaliados quanto ao perfil de proteínas ligantes de calmodulina e revelaram a presença de miosina-IIB superexpressa no cérebro de diabéticos.

- A diferença na expressão da proteína e do RNAm da miosina-IIB foi comprovada pelas técnicas de *western blot* e PCR em tempo real.

- A análise da distribuição da miosina-IIB revelou que o córtex do cérebro de diabéticos apresenta-se fortemente marcado.

Capítulo 3

Efeito da suplementação de cálcio, zinco e vitamina E no estresse oxidativo e na expressão de miosinas no cérebro de ratos diabéticos induzidos por estreptozotocina

1. ARTIGO CIENTÍFICO

PLoS Biology (fator de impacto: 12,68)

MYOSINS ARE DIFFERENTIALLY EXPRESSED UNDER OXIDATIVE STRESS IN STREPTOZOTOCIN-INDUCED DIABETES RAT BRAINS

Luciana Karen Calábria[1], Alice Vieira da Costa[1], Renato José da Silva Oliveira[1], Simone Ramos Deconte[1], Rafael Nascimento[1], Washington João Carvalho[1], Vanessa Neves de Oliveira[1], Carlos Alberto Arcaro Filho[2], Luciana Augusto de Rezende[2], Luiz Ricardo Goulart[1], Foued Salmen Espindola[1]

[1]*Institute of Genetics and Biochemistry, Federal University of Uberlândia, Campus Umuarama, 38400-902, Uberlândia-MG, Brazil.*

[2]*Department of Chemistry, Physic and Mathematic, University of Ribeirão Preto, 14096-900, Ribeirão Preto-SP, Brazil.*

Abstract

Diabetes mellitus is a chronic disease characterized by persistent hyperglycemia, which may lead to brain tissue damage due to oxidative stress. The enhancement of free radicals during hyperglycemia contributes to increased neuronal death and changes in synaptic transmission. This study evaluated the effect of oxidative stress and the use of antioxidants (calcium, zinc and vitamin E) supplementation on myosins expression levels in brain tissues of streptozotocin-induced diabetes experimental rat model. Lipid peroxidation, antioxidant enzymes activities and myosins IIB and Va expressions at transcriptional and protein levels were examined after 5 and 90 days post streptozotocin induction. The chronic effect of the streptozotocin-induced diabetes led to upregulation of glutathione peroxidase (GPx) and malondialdehyde (MDA) levels, and downregulation of superoxide dismutase (SOD) and catalase (CAT) activities. These alterations were accompanied by increased myosin-IIB and decreased myosin-Va expressions in diabetic rat brains. The antioxidant supplementation protected against hyperglycemia damage by reducing CAT activity and increasing GPx and SOD activities, although it had no influence on MDA levels. Interestingly, only diabetic rats restored myosin-IIB expression at both transcriptional and protein levels in brain tissues after supplementation, and no differences were observed in myosin-Va expression in comparison to non-diabetic rat brains. The functional alterations of antioxidant enzymes and myosins IIB and Va in rat brains of experimentally-induced diabetes were strongly associated with the disease and aging during acute and chronic stages, but only myosin-IIB expression could be reversed by antioxidants. These data highlight the supplementation (calcium, zinc and vitamin E) antioxidants effects on the oxidative stress and the involvement of myosins IIB and Va in the diabetes stages; however, their mechanisms of action on enzymes activities, lipid peroxidation and myosins expression remain to be elucidated.

Introduction

Diabetes mellitus is a multifactorial disease characterized by chronic hyperglycemia resulting from abnormalities in insulin action and/or insulin secretion [1]. Research evidences support that both acute and chronic hyperglycemia produce negative impacts on central nervous system leading to tissues damage [2,3]. One mechanism behind this neuronal injury is oxidative stress, due to excessive free radical generation from the oxidation of elevated intracellular glucose levels [4].

Brain contains large amounts of enzymes to protect against oxidative damage [5]. Endogenous antioxidant system, including enzymatic (glutathione peroxidase, superoxide dismutase, and catalase) and non-enzymatic (vitamin E, vitamin C, glutathione and uric acid) antioxidants, offers protection to cells and tissues against glucose-induced oxidative injury in diabetics [6,7,8,9,10].

The enhancement on oxygen free radical in brain during hyperglycemia [11] contributes to increased neuronal death trough protein oxidation, DNA damage, and peroxidation of membrane lipids [12] as well as changes in synaptic transmission. These alterations could lead to abnormal synaptic plasticity and cognitive impairments observed in experimental diabetes [13,14,15,16,17,18].

Myosins are a family of molecular motors that contains many classes and isoforms, which differ in their cellular distribution and function [19,20,21]. Among the myosin classes identified so far, the family classes II and V have been best characterized in neurons and are implicated in a wide variety of cellular functions in nervous system, including neuronal migration, growth cone motility, neuronal morphogenesis, axonal transport, and synaptic and sensory functions [22]. There are three isoforms of the non-muscle myosin II: IIA, IIB and IIC [20,23,24,25] and the myosin-V has been classified into subtypes Va, Vb and Vc [20,26]. The expression of myosin isoforms and its function in presynaptic terminals differs with type of neurons and properties of neurotransmitter release [27].

The rationale for using certain vitamins and minerals, or natural antioxidants, in the prevention and management of diabetes, is largely based on animal experiments and epidemiologic studies [28,29,30,31]. Diabetes is

associated with increased oxidative stress, and this fact, raises the interest of using antioxidant supplements in individuals with diabetes in an attempt to prevent long-term complications [32].

The present study aimed to evaluate the effect of oxidative stress and the use of antioxidants (calcium, zinc and vitamin E) supplementation on myosins expression in brain tissue of streptozotocin-induced diabetes experimental rat model during acute and chronic hyperglycemia.

Results

Blood glucose levels and body weight

Blood glucose and body weight (n = 8 rats/subgroup) were measured (Table 1). Streptozotocin injection produced diabetic rats with consistent high levels of blood glucose. The diabetic and supplemented diabetic rats had significantly higher blood glucose levels ($p < 0.001$) and lower body weight ($p < 0.05$) than non-diabetic rats of 5 and 90 days groups. When time was compared, before and after treatment, diabetic and supplemented diabetic rats showed decreased of body weight after treatment 5-day treatment; however, the non-diabetic group showed an increased body weight after the 90-day streptozotocin induction ($p < 0.001$).

Antioxidant defense system enzymes and lipid peroxidation

Glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) activities in brain of non-diabetic, diabetic and supplemented diabetic rats were presented in figure 1 (n = 4 rats/subgroup). Streptozotocin caused a noticeable diminution of cerebral GPx levels in diabetic rats at 90-day post induction ($p < 0.001$). Supplementation protected against hyperglycemic-induced reduction in GPx activity by maintaining the enzyme levels in the diabetic rat brains, similar to non-diabetic ($p > 0.05$) (Figure 1A). Otherwise, chronic effect of diabetes led to an upregulation of GPx levels ($p < 0.001$). No difference was observed between non-diabetic, diabetic and supplemented diabetic rats at the 5th day post induction.

Cerebral SOD activity was higher in diabetic rats than non-diabetic and supplemented diabetic rats at both 5-day ($p < 0.001$) and 90-day ($p < 0.05$)

periods. On the other hand, chronic effects of diabetes, decreased SOD activity ($p < 0.05$), and no change was observed on the non-diabetic animals (Figure 1B).

Figure 1C shows that cerebral CAT activity was significantly elevated in supplemented diabetic rats 5 days post induction when compared to the non-diabetic and diabetic rats in the same period ($p < 0.05$). Although, CAT activity was lower in rats at the 90-day, than rats at the 5-day period ($p < 0.05$), CAT levels were kept elevated in diabetic rats when compared to non-diabetic and supplemented rats. It is noteworthy that CAT levels were lower in supplemented diabetic than diabetic rats of 90 days post induction ($p < 0.05$), although this has not been observed in rats at the 5-day period. Besides, the increase in CAT activity in diabetic rats was accompanied by a significant decrease in the GPx activity in brain.

As presented in Figure 1D, the levels of malondialdehyde (MDA) were markedly elevated in diabetic rats at the 5-day period ($p < 0.005$), but supplementation of diabetic rats, had no influence on MDA levels. Moreover, aged non-diabetic rats had higher MDA levels than young non-diabetics ($p < 0.05$). No difference was observed between rats at the 90-day period and supplementation seems to be inefficient and does not affect the antioxidant status of both groups.

Determination of changes in myosins protein expression levels

In order to check if the myosin proteins levels could be altered after 5 days and 90 days of diabetes or supplementation duration, the amount of myosins IIB (210 kDa) and Va (190 kDa) were estimated by Western blotting ($n = 3$ rats/subgroup). Figure 2 displays the combined results of immunoblots and densitometrically quantitated myosins immunoreactivity represented as percentage of non-diabetic from diabetic and supplemented diabetic rats.

In a general manner, diabetes increased myosin-IIB protein levels in brain ($p < 0.05$). In diabetic rats of the 5-day group, myosin-IIB levels were 16.5% higher than non-diabetic values, and 36.8% in diabetic rats at the 90-day period. Myosin-Va protein levels have decreased 18% and 16% in diabetic rat brains as compared to non-diabetic rats in both 5- and 90-day periods, respectively ($p <$

0.05). No significant differences were observed for myosins protein levels in supplemented diabetic in both periods, except for myosin-IIB after the 5-day period, in which antioxidant supplementation has restored the protein content in the brain tissues to non-diabetic levels.

Determination of changes in myosins mRNA expression

Real time PCR (*n* = 3 rats/subgroup) was performed to measure changes in myosins gene expression for both *MYH10* (myosin-IIB) and *MYO5A* (myosin-Va) at 5- and 90-day periods (Figure 4). Changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ method with beta-2-microglobulin (*B2M*) as an endogenous control. Diabetic showed increased *MYH10* mRNA expression as compared to non-diabetic at 5- and 90-day periods (~2-fold). *MYO5A* mRNA was decreased in diabetic as compared to non-diabetic in both 5-day (0.35-fold) and 90-days (0.7-fold) periods. Supplementation reduced *MYH10* mRNA expression in brains in both periods, conferring ~0.35-fold decrease compared to diabetic group without antioxidants. However, no significant differences in *MYO5A* mRNA expression was observed between diabetic and supplemented diabetic in both periods. Amplification efficiency was tested by standard curves for *Rattus novergicus B2M* (R^2 = 0.9997), *MYH10* (R^2 = 0.996) and *MYO5A* (R^2 = 0.9999) generated by plotting the value of C_T cycle *vs*. the log of plasmid concentration (from 10^3 to 10^6 copies).

Discussion

Streptozotocin-induced diabetes is a well-documented model of experimental diabetes in rats. It provides a relevant example of endogenous chronic oxidative stress as a result of hyperglycemia [33]. In the present study, streptozotocin treatment produced significant increase in blood glucose levels along with reduction in body weight. In addition, the acute effect of diabetes led to decreased body weight while the chronic stage did not affect this anthropometric parameter in diabetic rats. These results are in accordance with other studies, which showed that Diabetes mellitus increase plasma glucose levels and decrease body weight of diabetic rats [34,35,36,37,38]. In fact, supplementation did not reduce blood glucose levels and this may have

contributed in part to the non-suppressive effect of antioxidants on oxidative stress, though the body weight had been reduced in diabetic rats.

We have also analyzed hyperglycemia-induced oxidative stress in rat brain. The increase in free radical generation along with depletion of antioxidants is the mechanism involved in diabetes-induced oxidative stress. There is evidence of alterations in free radical metabolism [39] and in the antioxidant parameters status [40,41] during diabetes in various tissues. Moreover, there are contradictory results in the literature regarding the effect of hyperglycemia-induced diabetes on antioxidant enzymes activities [42,43,44]. Thus, the current study show the effects of the concomitant use of vitamin E, calcium and zinc as antioxidants on the activities of defense enzymes, such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) in rat brains after 5 and 90 days of supplementation.

GPx and SOD are the first line of defense against free radical attacks. Their function is to catalyse the conversion of superoxide radicals to hydrogen peroxide [45]. Cerebral levels of GPx, a potent endogenous antioxidant, were reduced in diabetics at 90-day post induction. However, the activity of GPx has been shown increased in brain [36,46,47]. This increase was also observed after supplementation. GPx is responsible for decomposition of hydrogen peroxide and other lipid peroxides, and it is possible that supplementation have avoided the GPx activity reduction in induced hyperglycemia, maintaining the enzyme levels in brain of diabetics, similar to non-diabetic rats.

The increase in cerebral SOD activity was observed in diabetic rats at 5- and 90-day periods, which can lead to an important elimination of superoxide ions that inhibit the formation of hydroxyl radical in tissues. The increase SOD activity in type 2 diabetic mice brain has been reported [48,49] to be a putative protection mechanism of oxidative stress. In the meantime, the SOD activity also appears to be decreased in diabetic rats [36,50,51,52].

GPx activity in the supplemented diabetic rats remained at same levels as in non-diabetic rats, whereas the SOD activity increased significantly compared to non-diabetic. The alteration of antioxidant enzymes GPx and SOD levels in the diabetic rats could be attributed to peroxidative damage of the tissues caused by streptozotocin-induced hyperglycemia [53], while supplementation with antioxidants contributed to maintaining the optimum condition of enzyme

activity in the cellular organelles, by protecting them from peroxidation in acute diabetes.

Besides, the SOD and CAT are also the major antioxidant enzymes against oxidative stress, and appear to be decreased in diabetic rats [50,51]. CAT is responsible for the catalytic decomposition of hydrogen peroxide formed in cellular metabolism in oxygen and water molecules. Its increased activity at the early stage of the disease (5 days) during antioxidant supplementation followed by a decreased activity at the chronic stage (90 days), might indicate a fine modulation of the CAT activity in order to protect the brain against free radicals at acute stage of the diabetes and may also advocate for the importance of antioxidant supplementation at this stage for a better tissue response and protection. Simultaneous reduction in the activity of both SOD and CAT, likely makes the brain more vulnerable to hyperglycemia-induced oxidative stress. Although there are discrepancies in the levels of antioxidant enzymes reported in diabetic rats [54,55] our results have shown an important balance between GPx and CAT levels.

These antioxidant enzymes have a complementary catalytic activity leading to reduced MDA concentration, which represents lipid peroxidation products in tissue and blood. In the present study, we observed that MDA levels were significantly higher in diabetic rats at a 5-day period, corroborating with previous results [56]. Nevertheless, supplementation was not sufficient counteract those elevated levels of lipid peroxidation and did not protected the brain against the cytotoxic action and oxidative stress due to diabetes. It is well known that hyperglycemia increases lipid peroxidation, which may contribute to long-term tissue damage [57]. Besides, brain seems to be more sensitive than the other tissues and hence the increase in GPx activity was not sufficient to reduce MDA concentrations and thus to protect this tissue from lipid peroxidation.

Minerals (calcium and zinc) and vitamin E were added in diet of the diabetic rats to act as an antioxidant supplement in animals. Thus, our aim was to evaluate the effects of this supplementation on brain. Evidence from clinical studies has suggested that calcium could indirectly affect glucose metabolism, which would be desirable for diabetes prevention [58,59]. Zinc acts as an antioxidant in order to reduce oxidative stress, is essential for the function of

SOD [60], and is also involved in insulin synthesis [61] which are alterated in diabetes [62,63]. Treatment with zinc significantly reduces astrocytosis [64], elevates SOD activity [65], and may be able to prevent diabetes effects in brain or against various damaging effects, including oxidative injuries [66] and apoptosis [67,68,69]. Additionally, vitamin E possesses antioxidant activity [1,70,71], neuroprotective action [72], and plays a role in hyperglycemia prevention [73,74]. Moreover, treatment with vitamin E reduces GPx levels and lipid peroxidation in brain [11]. In contrast, there are evidences that excessive dietary zinc and vitamin E intake can induce pathological conditions associated with oxidative stress [75,76,77,78]. In regard to diabetes prevention through supplementation with micronutrients, the current evidences do not allow any particular recommendation for mineral or vitamin supplementation on a large scale. Given that diabetes is a condition of increased oxidative stress, antioxidant therapy may represent a potential coadjuvant to antidiabetic pharmacological treatment by improving the glucose metabolism [32].

On the other hand, our results have evidenced that chronic effect of diabetes in brain led to an upregulation of GPx levels, and downregulation of SOD and CAT activities, as well as a higher lipid peroxidation levels in old non-diabetic than in young non-diabetic rats. Our findings are consistent with previous reports [79,80,81,82], although another study has revealed that GPx and CAT activities are relatively unaffected by age [80].

Recent studies have shown that peroxidative damage to lipid and protein occurs with the aging process and the products of these reactions accumulate in the brain during aging [49,81,82,83,84]. Moreover, the brain is susceptible to oxidative stress, which is associated with age related brain dysfunction, due to its high content of key compounds for oxidative damage and antioxidant defense systems [85]. Thus, changes in the activities of oxidative enzymes seem to be more severely affected in the brain during the aging process. Evidence in literature suggests that there is a functional association between oxidative damage and brain dysfunction [86].

Both, aging and diabetes affect cognition, synaptic plasticity and neurotransmission in rats, hence the effects of diabetes and aging interact [87]. The impact of this interaction on the central nervous system is well recognized such as neurophysiological and structural changes associated mainly with

cognitive deficits and Alzheimer disease [14]. Degenerative changes of neurons and glia have been reported, and suggested that alteration in synaptic transmission could contribute to cognitive impairments observed in diabetics [17,18,37]. Besides, oxidative stress induced by hydrogen peroxide induces cytoskeletal reorganization and significantly enhances association of myosin to actin filaments [88]. Myosin has also been reported to be a particularly sensitive target of oxidative damage [89,90,91], although a direct link between oxidative stress and myosin dysfunction has yet to be established.

Several lines of evidence suggest that myosins may be involved in the regulation of synaptic vesicles. Myosin-IIB modulates neurotransmitter release from synapses [92] while myosin-Va mediates synaptic vesicle trafficking [26]. Here we show an increased protein expression of myosin-IIB, but a decreased of myosin-Va expression in diabetic rat brains. However, only diabetic rats restored protein and mRNA of myosin-IIB content to non-diabetic levels in brain after supplementation, and no differences were noted for myosin-Va levels in this condition. The increase in *MYH10* mRNA and decrease *MYO5A* mRNA in diabetic indicates that the functional alterations of myosins in brain may be associated with aging, in which this myosins are expressed at higher levels in adult stage than in early embryos and the expression increases with development [24]. Moreover, the alterations in myosin protein and mRNA levels may be linked with diabetes, even in transcriptional and translational levels, during acute and chronic diseases.

Myosins have a domain motor that binds to actin and ATP [93], and are modulated by ATPase activity [94]. The administration of streptozotocin reduces ATP concentration in rats [95], and this modifies the activity of various ATP-dependent proteins [96] like myosins. Therefore, the diabetes induction in rat brains by the administration of streptozotocin may influence on the myosins associated-ATP activity.

Diabetes mellitus studies have shown changes in expression or levels of some myosins, as *MYO9B* in intestinal permeability [97], and myosin-V immunoreactive myenteric neuronal density [98] in diabetic rats. However, no evidences have been reported before about different expression profiles of myosins IIB and Va in rat brains supplemented with antioxidants in acute and chronic diabetes. We cannot exclude the possibility that other translational

alterations of myosin related to the redox state of the cell contribute to functional impairment of myosin.

In agreement with the notion that unregulated Ca^{+2} release from the endoplasmatic reticulum to the cytoplasm due to hyperglycemia might be a mediator of beta cell dysfunction and apoptosis in diabetes, it is well recognized that cytoplasmic Ca^{+2} overload is an ubiquitous cause of cell death in neurons [99,100]. This Ca^{+2} influx leads to the activation of proteases like calpain [101] and the overactivation of Ca^{+2}-calpain pathways also contributes to apoptosis in diabetes [102]. In vitro studies have shown that purified myosin-Va is proteolyzed by calpain, producing two peptides of 130 kDa and 80 kDa [103]. Cleavage is at the PEST site located in the tail domain [104]. It is possible this mechanism of Ca^{+2} influx, following the activation of calpain and clivage of myosin had led to irreversible decrease of myosin-Va levels in diabetic rat brains. Otherwise, myosin-IIB lacks this PEST sequence and levels may be increased in diabetic as a compensatory mechanism.

In conclusion, antioxidant supplementation, calcium and zinc minerals, and vitamin E, strengthened the protection mechanisms of rat brains in experimentally-induced diabetes with increased of GPx activity and decreased CAT levels after 90 days post induction, but it did not protect against the oxidative damage at the chronic stage. Moreover, supplementation restored myosin-IIB protein and mRNA levels after 5 days, but had no effect on the myosin-Va levels in the brain in both acute and chronic stages.

The precise mechanism of the observed calcium, zinc and vitamin E mediated regulation of enzymatic activities, lipid peroxidation and expression of myosins cannot be ascertained from this study and remains to be explored in the future. Considering the brain is a heterogenous tissue and it is composed of different cell types and diverse functions, it is important to further detail the modulation of antioxidant enzymes and myosins expression in the different brain regions during acute and chronic diabetes. Therefore, this study contributes to giving some insights between the association and alterations in expression levels of myosins and antioxidant enzymes in the different regions of the diabetic nervous system.

Material and Methods

Animals

All experimental procedures were conducted in accordance with the ethical principles of the Brazilian Academy of Animal Experimentation and approved by the Committee of Ethics in Animal Experimentation from the University of Ribeirão Preto, UNAERP (066/09). Forty-eight male Wistar rats *Rattus norvegicus* (weight: 200–290g) were housed under standard conditions (22±1°C, humidity 60±5%, 12 h light/12 h dark cycle) with food and water *ad libitum* on the Central Biotery of UNAERP.

Induction of diabetes mellitus

After one week of acclimatization, the rats were subjected to a 24-h starvation. The animals were then anesthetized by intraperitoneal injection of xylazine/ketamine (1:1 v/v), and then streptozotocin (40 mg/kg body weight; Sigma-Aldrich), freshly dissolved in 0.01 M citrate buffer, pH 4.5, was injected into the penile vein (2 mL/kg). Food was denied for 90 min post injection. At 10 days after the streptozotocin or buffer injection, blood glucose was determined and animals with blood glucose above 200 mg/dL were scored diabetic. Animal (n = 48) weight was monitored daily until decapitation and surgical removal of brains 5 or 90 days after diabetes induction or supplementation.

Group distribution and rats supplementation

The rats were distributed randomly in three groups (n = 8, each): non-diabetic (ND), diabetic (D), and diabetic supplemented (SD). All animals were fed diets based on a modified AIN93G rodent diet, except supplemented diabetic group that was fed with additional calcium (2.5-fold), zinc (500 mg), and vitamin E (20-fold), following principles of American Institute of Nutrition [105].

Sample collection and tissue preparation

The brains of all animals were quickly removed, washed with chilled normal saline and immersed in liquid nitrogen. Simultaneously, the blood was also collected from the portal vein to confirm the glucose levels. For oxidative stress markers and western blotting analyses, each brains was individually

homogenized on ice in homogenization buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM EGTA, 5 mM ATP, 2 mM dithiothreitol, 1 mM benzamidine, 0.5 mM phenylmethane sulfonyl-fluoride, 0.1 M aprotinin, 20 μg/mL leupeptin, 0.1 mM pefabloc). The homogenates were centrifuged at 15,000 x *g* for 30 min at 4ºC and total protein concentration in the supernatant samples was measured following the Bradford assay [106].

Oxidative stress markers analysis

GPx and SOD activities: Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were measured using a commercial kit (Ransel and Ransod, Randox Laboratories Ltd., Crumlin, UK), following manufacturer's instructions. *CAT activity:* Catalase (CAT) activity was assessed spectrophotometrically monitoring hydrogen peroxide decomposition at 240 nm [107] and the substrate concentration was 20 mM for brain measurements. *Determination of lipid peroxidation product:* Lipid peroxidation in tissue was determined by measuring the presence of malondialdehyde (MDA) using the thiobarbituric acid test (TBARS), a commercial kit (Cayman Chemical Inc., MI, USA), following manufacturer's instructions.

Western blotting

Aliquots of supernatant samples were solubilized in a small volume of electrophoresis sample buffer containing an additional 100 mM Tris-HCl, pH 8.0, and 25% glycerol. Supernatant samples containing 30 μg of protein were analyzed by SDS-PAGE with a 5-22% acrylamide gradient and gels were electroblotted on nitrocellulose membranes in Tris-glycine buffer [108]. Blots were incubated with 5% dried milk in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), then probed with anti-myosin-IIB and anti-myosin-Va primary antibodies diluted to 0.2 μg/mL. The polyclonal antibodies were generated in rabbits against non-muscle myosin II purified and myosin-V head domain from chicken brain, and purified by affinity to antigen immobilized on nitrocellulose strips as described previously [109,110,111]. Following washes with TBS-T, blots were incubated with a peroxidase-conjugated anti-rabbit IgG (GE Healthcare; diluted 1:2000) and washed several times with TBS-T. Antibodies bound to the membranes were visualized by chemiluminescence

after treatment with ECL™ (GE Healthcare) followed by exposure to Hyperfilm™ (GE Healthcare) following manufacturer's instructions. The intensity of the protein bands was analyzed and compared using Scion Image software, version Alpha 4.0.3.2 (Scion Corporation) and results were expressed as percentage of total content.

mRNA expression levels using qRT-PCR

Total RNA was isolated from non-diabetic, diabetic and supplemented diabetic brains separately using TRIzol™ reagent (Invitrogen) following manufacturer's instructions and then resuspended in DEPC-treated water. Its quality and quantity was established by reading the optical density of each sample at 260 and 280 nm using NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies) and agarose gel electrophoresis. One microgram of total RNA was reverse transcribed at 37°C for 1 h in a 20 µL reaction mixture containing the first strand buffer, 40 U Murine Monoley Leukemia Virus Reverse Transcriptase (Invitrogen), 0.25 mM of each dNTP, 10 U RNAsin (Invitrogen), 0.5 mM DTT, and 126 pmol hexamer random primers (Invitrogen). Standards curves were prepared by cloning PCR products of *MYH10*, *MYO5A* and the housekeeping beta-2-microglobulin (*B2M*) fragments using TOPO TA Cloning Dual Promoter Kit (Invitrogen). The recombinant plasmid DNA was isolated and sequenced using MegaBACE 1000 automatic sequencer (Molecular Dynamics). The sequencing reaction was carried out using the DyEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare) following manufacturer's instructions. The concentration of plasmid was measured and the copy numbers calculated [112]. Serial dilutions of each standard curves were made in the range 10 to 10^7 copies per µL for *MYH10*, *MYO5A* and *B2M*. The reaction efficiency was calculated according to the formula $E = (10^{-1/slope} - 1) \times 100$, where the log of the each dilution was plotted with ΔC_T of housekeeping and target genes, $R^2 \geq 0.99$ and slope value about -3.32 were admitted to reaction efficiency of 100%. The qPCR assay was performed by using the 7300 Real-time PCR System (PE Applied Biosystems) using SYBR Green qPCR Master Mix reagent (Fermentas). The thermal cycling profile used was the Universal Program (PE Applied Biosystems). Primers were designed between exons junctions to avoid amplification of contaminating genomic DNA using Primers

Express software (PE Applied Biosystems). For *B2M* fragment amplification, the set of primers were: 5'-CGT CGT GCT TGC CAT TCA-3' and 5'-TCC TCA ACT GCT ACG TGT CTC AG-3'. The *MYH10* forward and reverse primers were respectively: 5'-CCA TGC CGG AGA ACA CAG T-3' and 5'-AAG CCC AGA CCA AAG AGC AG-3'. The *MYO5A* forward and reverse primers were respectively: 5'-ATT GAG GCT CGC TCT GTG GA-3' and 5'-ACG CAA AGT GGA TGA GCA GA-3'. The relative expression of each specific product was calculated by $2^{-\Delta\Delta CT}$ (C_T = fluorescence threshold value; ΔC_T = C_T of the target gene - C_T of the reference gene (*B2M*); $\Delta\Delta C_T$ = ΔC_T of the target sample - ΔC_T of the calibrator sample). All samples were run in duplicates. For expression analysis of the *MYO5A* gene, the cDNA was 4-fold diluted.

Statistical analysis

All values obtained are expressed as mean ± SEM). Data were initially analyzed by one-way analysis of variance (ANOVA) using the SigmaStat 3.5 software (Systat Software Inc.). When differences were detected by ANOVA, these sets of non-diabetic, diabetic and supplemented diabetic rats were compared using Student's *t*-test or Tukey's test to determine the statistical significance, which was assumed to be different when the comparison showed a significance level of $p < 0.05$.

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Figures and Legends

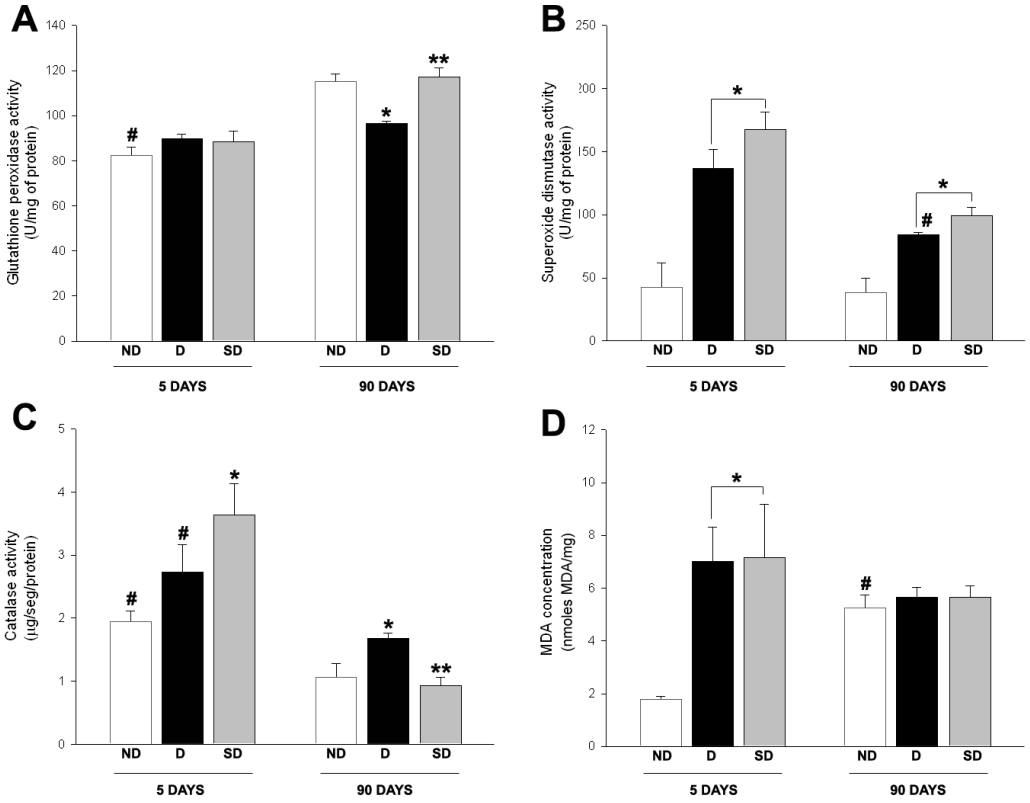

Figure 1 – Supplementation effects on antioxidant enzymes activities and lipid peroxidation in rat brain after 5 and 90 days treatment. Data are expressed as mean ± SEM, *n* = 4 rats/subgroup. **A**- glutathione peroxidase; **B**- superoxide dismutase; **C**- catalase; **D**- MDA concentration. Significant differences: *compared to non-diabetic (**ND**) (**A**- $p < 0.001$; **B; C-** $p < 0.001$ to 5 days, $p < 0.05$ to 90 days; **D-** $p < 0.05$); **diabetic (**D**) *vs*. supplemented diabetic (**SD**) (**A**- $p < 0.001$; **C**- $p < 0.05$); **#** 5 days *vs*. 90 days periods (**A**- $p < 0.001$; **B**, **C**- $p < 0.05$; **D-** $p < 0.05$).

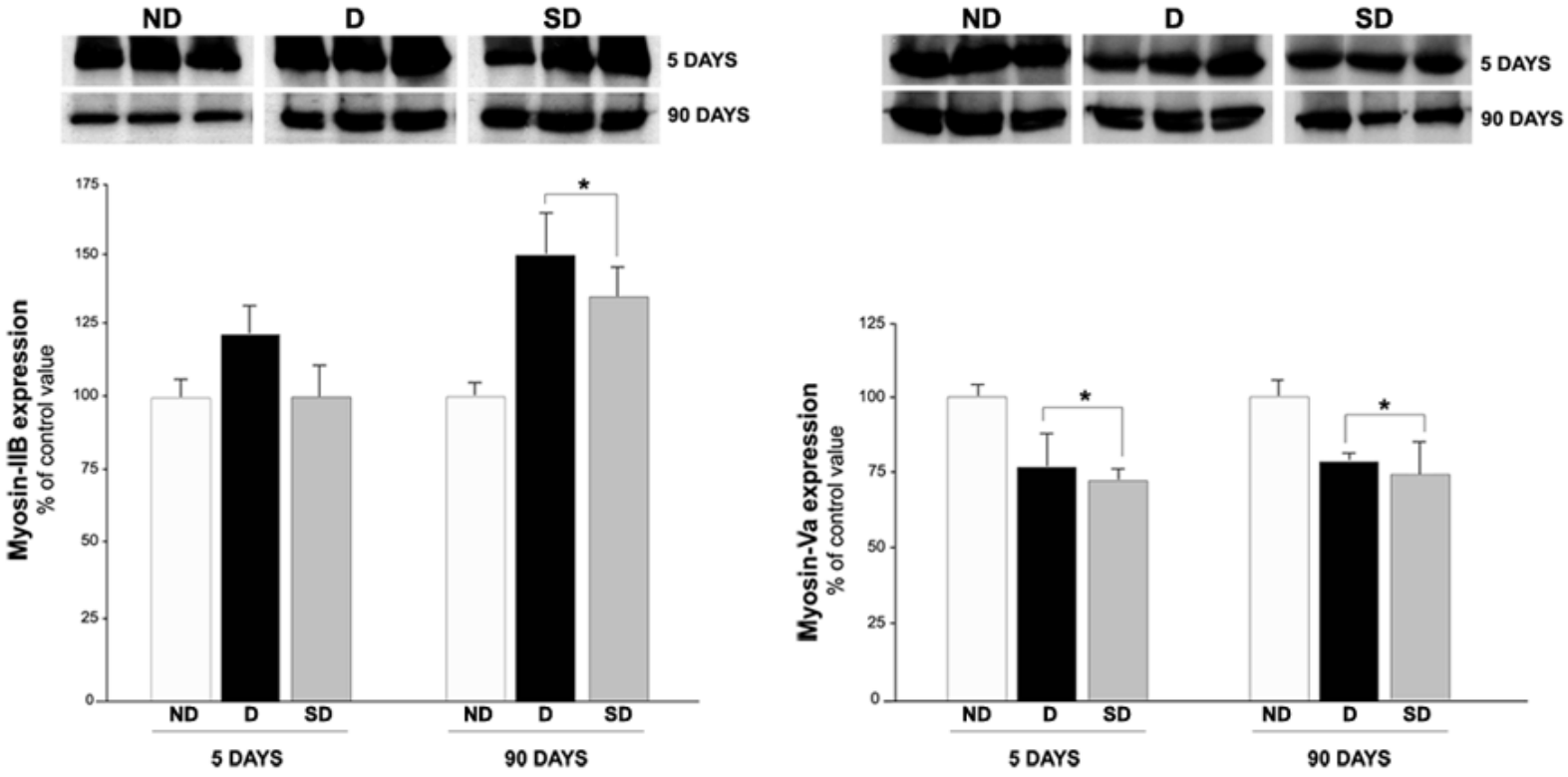

Figure 2 – Supplementation and diabetes effects on myosin-IIB and myosin-Va protein levels in rat brain after 5 and 90 days treatment. Western blots and quantification of myosins expressions from supernatant brain samples of non-diabetic (**ND**), diabetic (**D**) and supplemented diabetic (**SD**) rats after 5 and 90 days. The amount of myosin proteins showed on the immunoblot was determined densitometrically and expressed as a related percentage of the groups (n = 3 rats/subgroup). Values represent mean ± SEM. (*) denote a statistically significant ($p < 0.05$) difference as compared to **ND**.

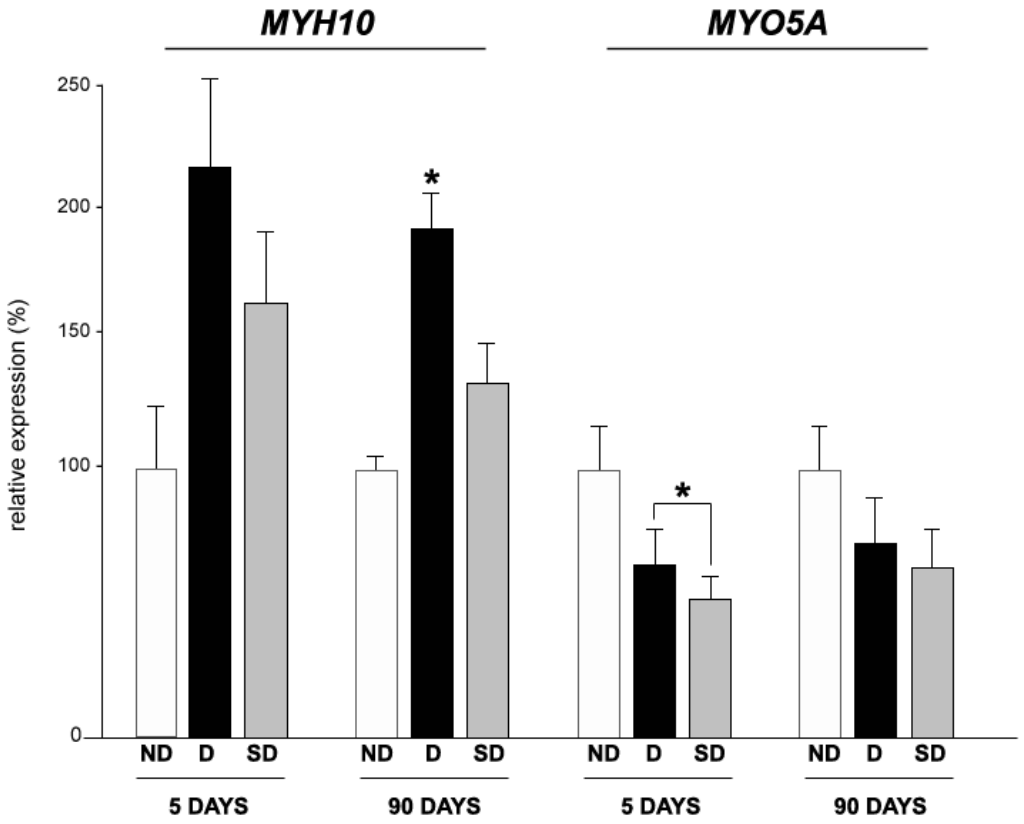

Figure 3 – Diabetes and supplementation effects on *MYH10* and *MYO5A* mRNA expression in rat brain after 5 and 90 days. Real time PCR and relative expression of *MYH10* and *MYO5A* mRNAs in brain samples of diabetic (**D**) and supplemented diabetic (**SD**) compared to non-diabetic (**ND**) rats after 5 and 90 days. Values represent mean ± SEM. (*****) denote a statistically significant ($p < 0.05$ to *MYH10* and $p < 0.01$ to *MYO5A*) difference as compared to **ND**; ($n = 3$ rats/subgroup).

Table 1. Effect of injection streptozotocin and supplementation on blood glucose and body weight after 5 and 90 days (n = 8 rats/subgroup).

Parameters	5 DAYS			90 DAYS		
	ND	**D**	**SD**	**ND**	**D**	**SD**
Blood glucose (mg/dL)						
Initial	92.25±1.69	447.13±15.60[a]	430.38±10.55[a]	89.25±2.28	398.88±19.09[a]	552.38±2.38[a]
Final	91.75±1.53	489.50±27.05[a]	461.00±29.45[a]	98.38±1.86	362.25±38.45[a]	467.13±39.39[a]
Body weight (g)						
Initial	252.5±7.61	236.75±8.04	245.63±6.41	257.50±3.32	249.88±4.38	221.75±4.48
Final	243.13±9.22	194.88±8.96[b,c]	199.75±8.15[b,c]	362.38±18.55[c]	222.63±25.69[b]	253.75±31.15[b]

Data are expressed as means ± S.E.M. Statistical analysis was done within days-groups.
ND, non-diabetic; **D**, diabetic; **SD**, supplemented diabetic; **5 days** and **90 days** diabetic or supplementation periods.
[a] $p < 0.001$, significant vs. ND.
[b] $p < 0.05$, significant vs. ND.
[c] $p < 0.001$, initial vs. final

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2. CONCLUSÕES

- O efeito crônico do diabetes levou ao aumento dos níveis da atividade da glutationa peroxidase e de malondialdeído, como também à diminuição da atividade das enzimas superóxido dismutase e catalase no cérebro de ratos diabéticos.

- As alterações nos marcadores enzimáticos e de peroxidação lipídica do estresse oxidativo estão acompanhadas por um aumento na expressão de miosina-IIB e diminuição de miosina-Va.

- A suplementação dos ratos diabéticos com uma ração enriquecida com antioxidantes minerais (cálcio e zinco) e vitamina E protegeu o cérebro de possíveis danos oxidativos consequentes da hiperglicemia, reduzindo a atividade da catalase e aumentando os níveis de glutationa peroxidase e superóxido dismutase, embora nenhuma influência tenha sido observada nos níveis de malondialdeído.

- Quanto à expressão de miosinas, somente a da classe II reestabeleceu os seus níveis no cérebro de ratos diabéticos após a suplementação.

- De forma geral, as alterações das enzimas antioxidantes e mosinas IIB e Va no cérebro de ratos diabéticos estão associadas com os estágios agudo e crônico do diabetes.