Heparin modulates the expression of genes encoding pro and anti-apoptotic proteins in endothelial cells exposed to intestinal ischemia and reperfusion in rats[1]

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ABSTRACT

PURPOSE: To investigate if expression of genes encoding pro and anti-apoptotic proteins in the rat enteric endothelial cells stimulated by intestinal ischemia followed by reperfusion (IR) can be modified by treatment with heparin (HP).

METHODS: Eighteen adult Wistar rats were divided in three groups: sham group submitted to laparotomy only (SG), ischemia followed by reperfusion group (IRG); ischemia followed by reperfusion plus pretreatment with HP 100 $mg.kg^{-1}$ (IRG+HP). Ischemia was performed by clamping of the superior mesenteric artery. After 60 min of ischemia, metal clamps were removed for reperfusion for 120 min. Gene expression of encoding pro (Casp1, Casp6, Casp3, Cflar, Fas and Pgl) and anti-apoptotic (Bcl2, Bcl2l1 and Naip2) proteins in rat enteric endothelial cells was evaluated by PCR microarray method.

RESULTS: Compared to rat endothelial cells of SG, the expression of pro-apoptotic genes was up-regulated in IRG while anti-apoptotic genes were down-regulated. In contrast, the expression of anti-apoptotic genes in IRG+HP was up-regulated while pro-apoptotic genes was down-regulated compared to SG.

CONCLUSION: The attenuation by heparin of intestinal ischemia-reperfusion previously demonstrated in rodents could be related with ability of this drug to stimulate and reduce gene expression of encoding anti and pro-apoptotic proteins, respectively.

Key words: Ischemia. Reperfusion Injury. Endothelial Cells. Gene Expression Profiling. Heparin. Rats.

Introduction

Ischemia followed by reperfusion (IR) triggers a multiple biological responses that cause tissue injury and loss of organ function. Although, oxygen is a critical substrate in the alleviation of ischemia, paradoxically, it also functions as a deleterious metabolite during the reperfusion of previously ischemic tissues[1]. It is important to note that IR injury is associated to organ transplants, major organ resections and in shock[2].

The oxidative stress caused by IR may lead either to immediate cell death (necrosis), controlled cell death (apoptosis) or trigger changes of the cell phenotype in response to IR injury[3,4]. Pro and anti-apoptotic proteins are involved in the cascade of IR injury[3,4]. Some studies have proposed that the use of the quantitative real time polymerase chain reaction method (qRT-PCR) to evaluate specific gene expression during IR could be crucial to understanding the biochemical mechanism and the effects of protective treatments[3-4]. It is assumed that proteins that function together in a pathway or structural complex are likely to evolve in a correlated fashion[3-4].

Pharmacological studies have proposed the use of different class of drugs to attenuate or prevent the structural and functional lesions caused by IR[5]. Recent studies have suggested that glycosaminoglycan heparin (HP) and its derivatives, agents currently used as anticoagulant and antithrombotic, act as free radical scavengers and inhibitors of apoptosis in several cell types, protecting them from IR injury[6-7]. The initial endothelial damage plays a main role in IR, but the limited access to endothelial tissue is a major constraint when investigating the cellular mechanisms of vascular inflammation[1-4].

Using Rat Endothelial Cell Biology PCR microarray, we decided to investigate if intestinal IR modifies gene expression encoding pro and anti-apoptotic proteins in the rat enteric endothelial cells and if HP treatment modifies this response.

Methods

All experimental protocols used in the present study were approved by the Ethics Committee in Research of the Federal University of Sao Paulo (UNIFESP) on according to the recommendations of the international legislations on animal protection (CEP 0362/11). The study was designed as a randomized controlled trial with a blinded assessment of the outcome.

Eighteen male Wistar-EPM1 rats weighed 250 to 300g were obtained of Center for the Development of Experimental Models for Medicine and Biology - CEDEME-UNIFESP). The animals were housed under controlled temperature and light conditions with a 12h light/dark cycle, and free access to water and standard pellet chow until 6h prior to the surgical procedures. All procedures were conducted in the laboratories of Animal Experimentation of Department of Surgery, Morphology, Biochemistry and Pharmacology of the UNIFESP.

The animals underwent anesthesia (intramuscular injection of 80 $mg.kg^{-1}$ ketamine and 10 $mg.kg^{-1}$ xylazine), median laparotomy, and exposure of the superior mesenteric vessels and were randomly divided in three groups:

Sham group (SG, n=6): These animals were submitted to laparotomy without clamping of superior mesenteric artery;

Ischemia and reperfusion group (IRG, n=6): These animals were submitted to laparotomy and clamping of superior mesenteric vessels for 60 min followed by 120 min of reperfusion; saline solution 0.9% was inject in the femoral vein 5 min before ischemia, 5 min after reperfusion and 55 min of reperfusion;

Ischemia and reperfusion and heparin group (IRG+HP, n=6): These animals were submitted to laparotomy and clamping of superior mesenteric vessels for 60 min followed by 120 min of reperfusion; HP 100 $mg.kg^{-1}$ was inject in the femoral vein 5 min before ischemia, 5 min after reperfusion and 55 min of reperfusion.

In all groups, after 180 min of IR, intestinal segments (3 cm) were removed at distance of 20 cm from the duodenum-jejunum flexure, opened longitudinally, gently washed in saline solution 0.9%, wrapped in aluminum foil, and immediately freezing in liquid nitrogen for storage in ultralow freezer. After this, euthanasia of animals was performed by intravenous injection of embutamide, mebezone and tetracain mixture.

The tissue samples from each group were prepared to assays using Endothelial Cell Biology Rat RT² Profiler™ PCR microarray method (SA Biosciences - Qiagen Co, USA). These assays were performed according to the manufacturer's protocol. Briefly, total RNA was extracted from tissues using Trizol reagent (Life Technologies, USA) and purified using an RNeasy MiniKit (SA Biosciences - Qiagen Co, USA). Concentration of each total RNA sample was determined by spectrophotometry, and the quality was assessed by electrophoresis on 2% agarose gels. The first strand complementary DNA (cDNA) was synthesized using 1 μg of total RNA and the RT² First Strand Kit (SA Biosciences - Qiagen Co, USA). Equal amounts of cDNA and the Master Mix SYBR® Green qPCR Mastermix (SA Biosciences - Qiagen Co, USA) were distributed to each well of the PCR microarray plate containing the pre-dispensed gene-specific primer sets. PCR was performed according to the manufacturer's instructions in 96-well plates to detect the expression of 84 genes related to oxidative

stress, five housekeeping genes (ACTB, Gapdh, Hsp90ab1, Hprt1, Gusb) used for normalizing the PCR microarray data, and one negative control for genomic DNA contamination. The negative control primer set specifically detects non-transcribed, repetitive genomic DNA with a high level of sensitivity. Three wells of reverse transcription controls (RTCs) were used to verify the efficiency of the RT reaction with a qPCR assay that specifically detects template synthesized from the RNA control of the first strand synthesis kit. The replicate positive PCR controls (PPCs) were used to determine the efficiency of the polymerase chain reaction itself. These controls use a pre-dispensed artificial DNA sequence and a specific primer set to detect it. The two sets of replicate control wells (RTCs and PPCs) also test for inter-well and intra-plate consistency. The instrument's software (MxPro Equipment Real Time Systems, Stratagene, GE, Co, USA) calculates the threshold cycle (Ct) values for all of the genes in the array. Finally, it performs pair-wise comparisons by calculating fold changes in gene expression from the raw threshold cycle data using the ΔΔCt method. The method used in our study to determine the relative expression levels of the genes of interest in each sample is contained in the spreadsheet for PCR Array Data Analysis v3.3 (SA Biosciences - Qiagen Co, USA).

Statistical analysis

The analysis of gene expression by real-time PCR represents a relative quantification of genes of interest. The control samples (SG) was used as reference baseline. The results were transformed into log2 scale for the calculation of averages and standard errors and for statistical analysis. Fold-change [2^ (- Delta Delta Ct)] is the normalized gene expression [2^ (- Delta Ct)] in the Test Sample (IRG or IRG+HP) divided the normalized gene expression [2^ (-Delta Ct)] in the Control Sample (SG). Fold-regulation represents fold-change results in a biologically meaningful way. It was considered that fold-change values greater than one indicates a positive or an up-regulation, and the fold-regulation is equal to the fold-change. The *p* values are calculated based on a Student's t-test of the replicate 2^ (-Delta Ct) values for each gene in the control group and treatment groups. P values less than 0.05 was considered significant in all experiments and indicated by symbols.

Results

Table 1 and Figure 1 shows the expression levels of ten genes related to rat enteric endothelial cell biology from SG, IRG, IRG+HP. Table 1 shows the lists of studied genes and its abbreviations (alphabetical order), descriptions and expression (up or down regulation).

TABLE 1 - Expression of ten genes related to enteric endothelial cell biology from rats of sham (SG), ischemia and reperfusion (IRG) and ischemic and reperfusion *plus* heparin (IRG+HP) groups. Significant values of fold up (+) or down (-) regulation was marked in bold [2^ (-Delta Ct].

#	Bank	Symbol	IRG	IRG+HP	P value
1	NM_017059	Bax	+1.48	**+9.69**	**0.000002***
2	NM_016993	Bcl2	**+3.86**	**+13.50**	**0.000003***
3	NM_031535	Bcl2l1	+1.01	**+22.89**	**0.000002***
4	NM_012762	Casp1	**+100.68**	**+17.94**	**0.000018***
5	NM_012922	Casp3	**+8.28**	+1.43	**0.007822***
6	NM_031775	Casp6	**+6.86**	**+3.55**	**0.000026***
7	NM_057138	Cflar	**+41.60**	+0.63	**0.000175***
8	NM_139194	Fas	**+23.73**	**+6.96**	**0.000007***
9	XM_226742	Naip2	+1.31	**+9.33**	**0.000087***
10	XM_574314	Plg	**+6.69**	**+10.85**	**0.000264***

(*)p≤0.05

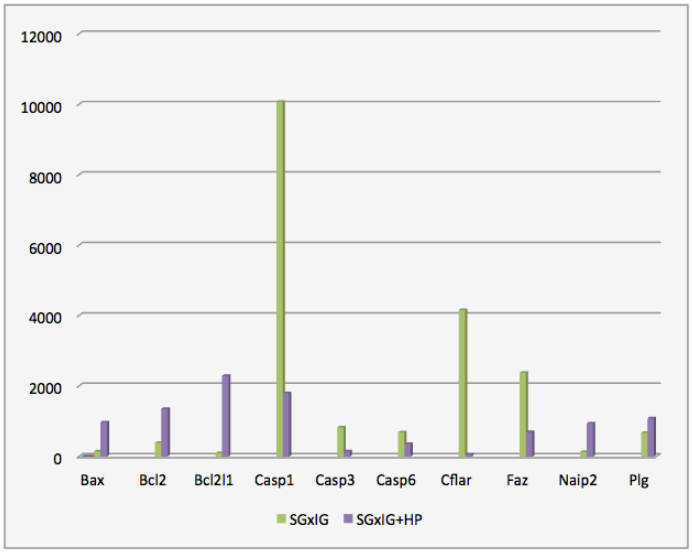

FIGURE 1 - Scatter plot showing up (+) and down (-) fold-regulation of gene expression related to endothelial cell biology from rats of sham group (SG) *versus* ischemic and reperfusion group (IRG) and sham group (SG) *versus* ischemic and reperfusion *plus* heparin group (IRG+HP).

Compared to SG, pro-apoptotic gene expression (Casp1, Casp3, Casp6, Cflar, Fas and Pgl) was up-regulated while anti-apoptotic gene expression (Bcl2, Bcl2l1 and Naip2) was down-regulated in IRG (increase of apoptosis). In contrast, anti-apoptotic gene expression was up-regulated while pro-apoptotic gene expression was down-regulated in IRG+HP.

Discussion

The complex mechanisms involving cellular death after IR injury are not fully understood. Significant increases of content of reactive oxygen species (ROS) in tissues exposed to IR have been observed. In these tissues were showed increment of hydroxyl radicals, superoxide anions, and hydrogen peroxide, associated with decreased antioxidant enzyme activities[8,9]. ROS play a major role in the pathophysiology of the ischemic injury via oxidative damage to membrane lipids and proteins[8,9].

Superoxide dismutase (SOD) represents the first line of defense against oxidative stress, by catalyzing the dismutation reaction of superoxide anion to hydrogen peroxide[8-10]. Malondialdehyde (MDA), one of the major products of lipid peroxidation, has been extensively measured as an index of this process[10]. In addition, intracellular Ca^{2+} overload destabilizes the cellular function and architecture, resulting in damage and eventual cell death[10]. This Ca^{2+} overload is caused by multiple mechanisms, but mainly by influx by voltage-gated Ca^{2+} channels and release from Ca^{2+} the intracellular organelles, such as endoplasmic reticulum[10].

In vascular endothelial cells, thrombin induces increment of cytosolic Ca^{2+} levels and subsequent nitric oxide (NO) production[11]. Thrombin cleaves protease-activated receptors resulting in activation of intracellular signals[11]. Ca^{2+} plays a unique role in the pathophysiology of ischemia, because it causes several damaging events by activating a variety of Ca^{2+}-dependent enzymes, including protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase, Ca^{2+}-dependent nitric oxide synthase, and calpain, as well as various proteases and endonucleases[10-12]. As a result of the formation of cytotoxic products, such as free radicals and leukotrienes, excess intracellular Ca^{2+} triggers irreversible mitochondrial damage, inflammation, and programmed cell death (apoptosis) and/or necrotic[10-12].

Using Endothelial Cell Biology Rat PCR microarray method, we showed that gene expression encoding of pro-apoptotic proteins (Casp1, Casp3, Casp6, Cflar, Fas and Pgl) was up-regulated while anti-apoptotic gene expression (Bcl2, Bcl2l1 and Naip2) was down-regulated in rats submitted to intestinal IR (Figure 1 and Table 1). These results indicate an increment of apoptotic process in endothelial cells injured by IR. In contrast, pretreatment with HP produced an increase of anti-apoptotic gene expression accompanied by reduction of pro-apoptotic gene expression in endothelial cells of rats submitted to IR (Figure 1 and Table 1), indicating that HP treatment is able to attenuate apoptotic process in response to intestinal IR injury.

It is well documented that HP and its derivates produce a protector effect in tissue/organs submitted to IR injury[6,7,11,13-18,20,21]. This effect appear be mediated by various cellular actions of HP and its derivates, including free radical scavenger and inhibition of apoptotic process in cell and tissues exposed to IR injury[6,7,11,13-18]. However, molecular mechanisms involved in these cytoprotector actions remain yet under investigation.

HP and its derivatives have long been proposed for stroke treatment. HP may prevent venous thromboembolic complications, improve neurologic outcomes, reduce mortality, and prevent early recurrence. Some low-molecular-weight HP (LMWH), as nadroparin and tinzaparin, has been tested in the treatment of acute IR lesions[13-18]. Studies published from 2009 to 2012 by Taha *et al*.[19] using animal model of hepatic IR showed that HP attenuated hepatic lesions caused by IR in rats and rabbits. For example, Taha *et al*.[19] showed that protective effects of HP on hepatic IR injury in rabbits could be partly mediated by controlling by HP of aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase levels. In addition, histological analysis showed significant reduction in cellular lesions caused by IR among livers of HP-treated rabbits.

Celik *et al*.[20] showed that HP treatment in animals submitted to IR attenuated flap necrosis and improved survival owing to radical scavenging, antioxidant effects, and supportive activities on capillary permeability and transudation. Medeiros *et al*.[21] showed that HP treatment in animals submitted to IR stimulated intracellular calcium release, activation of phospholipase C and of calcium calmodulin kinase II, NO production and vascular protective factors.

These results indicate that attenuation by HP of intestinal IR injury previously demonstrated in rodents could be related with ability of this drug to modulate expression of proteins involved in apoptotic response, by up-regulation of genes encoding anti-apoptotic proteins and down-regulation of genes encoding pro-apoptotic proteins.

Conclusion

The pretreatment with heparin was demonstrated in rodents that could be related with ability of this drug to stimulate and reduce gene expression of encoding anti and pro-apoptotic proteins, respectively.

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