Gene expression profile of oxidative stress in the lung of inbred mice after intestinal ischemia/reperfusion injury[1]

Adauto Tsutomu Ikejiri[I], Frederico Somaio Neto[II], José Carlos Chaves[III], Paulo Roberto Bertoletto[IV], Roberto Teruya[V], Eduardo Rodrigues Bertoletto[VI], Murched Omar Taha[VII], Djalma José Fagundes[VIII]

[I]Fellow Master degree in Surgery, Postgraduate Program in Interdisciplinary Surgical Sciences, Sao Paulo Federal University (UNIFESP), Sao Paulo-SP. Associate Professor, Medical School, Federal University of Grande Dourados (UFGD), Dourados-MS, Brazil. Intellectual and scientific content of the study, design the protocol, technical procedures, acquisition and interpretation of data, statistical analysis, manuscript writing.
[II]Fellow Master degree in Surgery, Postgraduate Program in Interdisciplinary Surgical Sciences, UNIFESP, Sao Paulo-SP. Associate Professor, Medical School, UFGD, Dourados-MS, Brazil. Technical procedures, acquisition and interpretation of data, statistical analysis, manuscript writing.
[III]Fellow PhD degree in Surgery, Postgraduate Program in Interdisciplinary Surgical Sciences, UNIFESP, Sao Paulo-SP. Associate Professor, Medical School, UFGD, Dourados-MS, Brazil. Technical procedures, acquisition and interpretation of data, statistical analysis, manuscript writing.
[IV]PhD, Associate Professor, Medical School, UFGD, Dourados-MS, Brazil. Technical procedures, acquisition and interpretation of data, statistical analysis, manuscript writing.
[V]Fellow PhD degree in Surgery, Postgraduate Program in Interdisciplinary Surgical Sciences, UNIFESP, Sao Paulo-SP. Affiliate Professor, Surgery Department, Federal University of Mato Grosso do Sul (UFMS), Campo Grande-MS, Brazil. Technical procedures, acquisition and interpretation of data, statistical analysis, manuscript writing.
[VI]Graduate student, Universitary Center Sao Camilo, Sao Paulo-SP, Brazil. Technical procedures, acquisition and interpretation of data.
[VII]PhD, Associate Professor, Surgical Techniques and Experimental Surgery Division, Surgery Department, UNIFESP, Sao Paulo-SP, Brazil. Manuscript writing, critical revision.
[VIII]PhD, Associate Professor, Surgical Techniques and Experimental Surgery Division, Surgery Department, UNIFESP, Sao Paulo-SP, Brazil. Intellectual and scientific content of the study, design the protocol, technical procedures, interpretation of data, manuscript writing, critical revision.

ABSTRACT

PURPOSE: To determine the gene expression profile associated with oxidative stress and antioxidant defense in the lung tissue of mice subjected to intestinal ischemia and reperfusion.

METHODS: Twelve male, inbred mice (C57BL/6) were randomly assigned to one of two groups. The control group (CG) underwent anesthesia and laparotomy and was observed for 120 minutes; the ischemia/reperfusion group (IRG) was subjected to anesthesia, laparotomy, and ischemia of the small intestine for 60 minutes and to 60 minutes of reperfusion. A pool of six mice from each group was subjected to a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to analyze the oxidative stress and antioxidant defense genes. All genes that were up-regulated or down-regulated greater than three-fold, based on the algorithm [2^(ΔΔCt)], were considered to be biologically meaningful.

RESULTS: Out of a total of 84 genes in the lung that are related to oxidative stress, 67 (79.7%) were up-regulated and 17 (20.2%) were down-regulated. Only two genes (2.3%), Lpo (lactoperoxidase) (+3.51) and Gpx4 (glutathione peroxidase) (+4.10), were expressed above the three-fold threshold, while none of the down-regulated genes were expressed outside of this threshold.

CONCLUSION: The intestinal ischemia/reperfusion injury promoted a gene expression profile consisting of the positive expression of oxidative genes in a remote organ. This suggests that activate signaling pathways are implicated in both cell survival and the maintenance of genome integrity in the lung.

Key words: Gene Expression. Oxidative Stress. Lung. Reperfusion Injury. Mice.

Introduction

Mesenteric ischemia is a severe disease of the gastrointestinal tract that can result in varying degrees of tissue damage, which results in intestinal necrosis. When therapeutic measures are performed to restore blood flow, the paradoxical worsening of ischemic damage by the action of cytokines, interleukins, and especially of reactive oxygen species (ROS) can occur[1,2]. Ischemia-reperfusion injury is often observed in abdominal organ transplantations, arterial and venous occlusive diseases of the digestive tract, and circulatory shock[3].

Ischemia and reperfusion injury of the intestine (IRI) is a clinical event that is associated with the development of systemic inflammation, dysfunction of the pulmonary microcirculation, and the outbreak of severe Acute Respiratory Distress Syndrome (ARDS). Reports demonstrate that individuals under IRI are subject to develop lung damage[4,5].

Research assessing the damage of remote organs affected by IRI has focused on the morphological and biochemical cascades involved in oxidative stress directly through ROS or indirectly through the products of lipid peroxidation[6-9]. The use of molecular biological techniques allows for the reliable assessment of large numbers of genes, and this is relatively simplified using reverse transcription-quantitative polymerase chain reaction (RT-qPCR)[10-12]. This tool is used to study the expression of genes involved in oxidative stress and antioxidant defense in remote organs to better understand the various aspects of this phenomenon in the areas of patient diagnosis, monitoring and therapy[13,14].

In this context, to search for a new approach to study ischemia and reperfusion, the aim of this study was to identify the expression profile of a set of genes already known to be involved in oxidative stress and antioxidant defense. The identification of the genes involved will eventually serve to establish methods to monitor the evolution of IRI in the organs that are primarily affected as well as in remote organs.

Methods

The experimental protocol (#0792/09) was approved by the Ethics Committee of the Federal University of Sao Paulo (UNIFESP) and the protocol (#306/08) was ratified by Ethics Committee of Federal University of Grande Dourados (UFGD), Mato Grosso do Sul, Brazil. Inbred mice (C57BL/6) were maintained according to the "Guide for The Care and Use of Laboratory Animals" (Institute for Laboratory Animal Research, National Academy of Sciences, Washington, DC, 1996). The study was designed to be a randomized controlled trial with a blinded assessment of the outcome.

We used 12 male, inbred C57BL/6 mice (Center for the Development of Experimental Models for Medicine and Biology - CEDEME-UNIFESP). The animals, weighing from 30 and 35 g, were housed under temperature- and light-controlled conditions and had free access to water and standard pellet chow until 6 hours prior to the experimental procedures. All procedures were conducted in the laboratories of Experimental Surgery, Department of Surgery, Federal University of Grande Dourados (UFGD).

Anesthesia and surgical procedures

Under general anesthesia (44 mg/kg of ketamine i.m., 2.5 mg/kg of xylazine i.m., and 0.75 mg/kg of acepromazine i.m.) and aseptic conditions, all 12 animals underwent a midline laparotomy. In six animals from the IRG (ischemia/reperfusion group), the superior mesenteric artery was carefully dissected and then occluded using a micro vascular clamp for 60 minutes. Ischemia was confirmed by observing the pale appearance of the clamped small bowel and the absence of beats in the mesenteric branches artery. After the clamp was removed, reperfusion was evaluated based on immediate color recovery and artery beats (x4 magnification device). The surgical wounds remained covered with wet gauze wrappings throughout the experiment to minimize evaporative loss. After 120 minutes, in the six animals of the CG (control group) without ischemia or reperfusion, or after 60 minutes following reperfusion in the animals of the IRG, the surgical wound was opened, and samples were collected from the right lung of all animals in both groups. The samples were wrapped in aluminum foil, immersed in liquid nitrogen, and sent for genomic processing to the Laboratory of Molecular Biology, Department of Gynecology (UNIFESP). After the samples were collected, the animals were sacrificed by decapitation.

RNA preparation (RT-qPCR)

The RT² Profiler™ PCR array from SA Biosciences (Frederick, Maryland; cat # PAMM-065) was used to analyze mouse oxidative stress and antioxidant defense pathways according to the manufacturer's protocol. Briefly, total RNA was extracted from the both CG group and the IRG group lung tissues using Trizol reagent (Life Technologies, Grand Island, NY, USA), and the RNA was purified (RNeasy Mini Kit, Qiagen Co, USA). The total RNA concentration was determined using spectrophotometry, and the quality was assessed by analyzing the

RNA on a 2% agarose gel. One microgram of total RNA was used to create the first-strand complementary DNA (cDNA) using a RT2 First Strand Kit (SA Biosciences). An equal amount of cDNA was mixed with SYBR Green reagent (SA Biosciences Qiagen, Co) and dispensed into each well of a PCR array plate containing pre-dispensed, gene-specific primer sets. PCR of 84 genes related to oxidative stress was performed in 96-well plates, according to manufacturer's instructions. Five housekeeping genes (Actin B, Gapdh, Hsp90ab1, Hprt1, and Gusb) were used to normalize the PCR array data, and one negative control was used to monitor for genomic DNA contamination. The negative control primer set specifically detects non-transcribed, repetitive genomic DNA with a high level of sensitivity. The PCR array also contained three wells of reverse transcription controls (RTC) to verify the efficiency of the RT reaction.

The qPCR assay specifically detects templates synthesized from the first strand synthesis kit's built-in external RNA control. The replicate positive PCR controls (PPC) were used to check the efficiency of the PCR. These elements use a pre-dispensed, artificial DNA sequence and the primer set that detects it. The two sets of replicate control wells (RTC and PPC) also test for inter-well and intra-plate consistency. The instrument's software (MxPro Equipment Real Time Systems, Stratagene, GE, Co) calculates the cycle threshold (Ct) values for all genes in the array. Finally, the software calculates fold changes in gene expression levels from the raw threshold cycle data using the ΔΔCt method for a pair-wise comparison. This method was used in our study to determine the relative expression levels of the genes of interest for each sample. The data were stored in a spread sheet for analysis using PCR Array Data Analysis v3.3 (SA Biosciences Qiagen, Co).

Statistical analysis

Gene expression data for each sample was evaluated in triplicate. Student's-test was applied to validate the homogeneity of the reaction of expression of each gene (p value<0.05). For the comparison between the two groups, the software calculated the variation in the cycle threshold (Ct) in the study group (IRG) compared to the cycle threshold (Ct) of the control group (CG), and this was expressed as a logarithmic base (2) value based on the formula [2^(ΔΔCt)]. The gene expression results are presented as positive/up-regulation expression (IRG>GC) or negative/down-regulation expression (IRG< CG). The numbers represent how many time seach gene was expressed above [plus sign (+)] or below [minus sign (-)]. The software established that the results three-fold above (over expression) or three-fold below (hypo expression) the threshold allowed by the algorithm [2^(ΔΔCt)] are biologically relevant.

Results

From the 84 genes investigated in lung tissue in the animals subjected to 60 minutes of small bowel ischemia and 60 minutes of reperfusion compared to control group without ischemia/reperfusion 67 (79.7%) had a positive expression, whereas 39 (46.4%) genes were significantly expressed. About 53 (63%) genes were up-regulated in the interval of one fold, where about 25 (29.7%) were significantly expressed. On the other hand, 12 (14.2%) genes were up-regulated in the interval of two folds, all of them significantly expressed. Two genes (2.3%) had a positive expression and significantly higher than three folds as allowed by the algorithm (Figure 1).

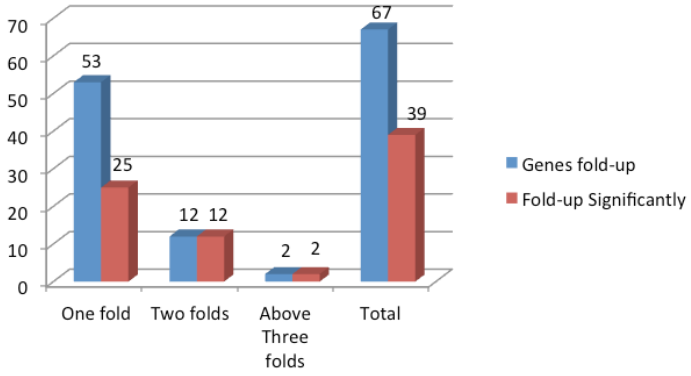

FIGURE 1 - The expression levels of 84 genes of lung tissue related to the oxidative stress and antioxidant defense in the inbred mice distributed by the numbers of fold-up regulation depicted by the intervals of one, two or three folds as allowed by the algorithm.

Considering the 84 genes investigated in lung tissue in the animals subjected to 60 minutes of small bowel ischemia and 60 minutes of reperfusion compared to control group without ischemia/reperfusion 17 (20.2%) had a negative expression. About 15 (17.8%) of genes were down-regulated in the interval of one fold, however only 2 (2.3%) were significantly expressed. On the other hand, 2 (2.3%) genes were down-regulated in the interval of two folds, all of them significantly expressed. No gene showed a negative expression over than three folds (Figure 2).

The scatter plot showed (Figure 3) the expression levels of the 84 lung tissue genes that are related to oxidative stress and antioxidant defense in the inbred mice. The blue arrow highlights the Gpx4 gene (+4.10) and the yellow arrow highlights the Lpo gene (+ 3.51) both significantly expressed three folds above the threshold (pink line) allowed by the algorithm.

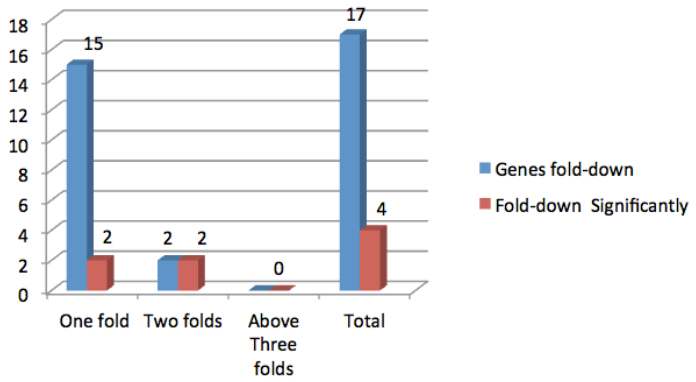

FIGURE 2 - The expression levels of 84 genes of lung tissue related to the oxidative stress and antioxidant defense in the inbred mice distributed by the numbers of fold-down regulation depicted by the intervals of one, two or three folds as allowed by the algorithm.

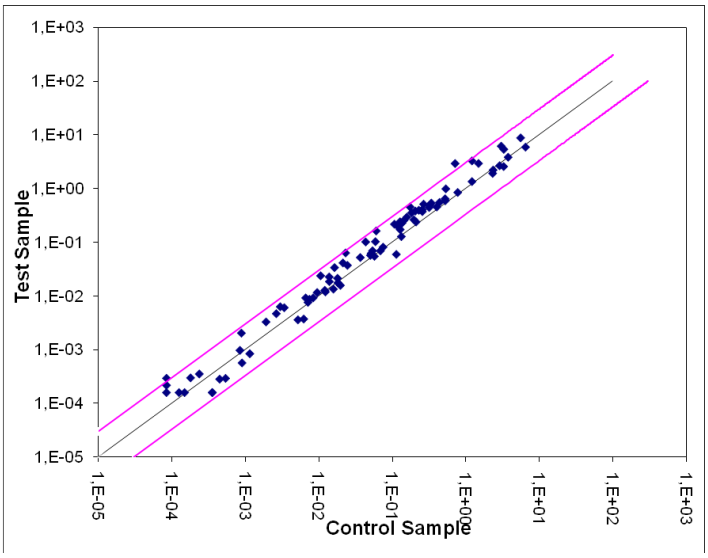

FIGURE 3 - Scatter plot of the up-regulation and down-regulation of all of the genes that were investigated. The black line indicates a fold change [(2^(ΔΔC_t)] of 1. The pink lines indicate a three-fold change in the gene expression threshold.

Discussion

Using an animal model to observe a short period (60 minutes) of reperfusion after a previous period (60 minutes) of ischemia in the intestine of inbred mice, we have shown that from a total of 84 genes associated with oxidative stress and antioxidant defense, 67 genes were positively expressed in response to the remote injury in the lung tissue (Figures 1 and 3). These events involved a number of genes that encode proteins known to be related to processes of production and modulation of reactive oxygen species and antioxidant defense. The local response to oxidative stress releases pro-inflammatory substances in to the blood stream that act remotely in other organs[4]. The lung is an organ that can be remotely impacted depending on the severity and the length of time of the deleterious stimulus shock in the ischemic organ[6-8].

The rationale for this research was to identify genes that are part of a standardized gene expression procedure[15], the expression of which changes due to remote tissue ischemia. This finding is based on the assumption that the expression of certain genes encode for proteins that protect the cell from any harmful effects[13,14].

Sixty-seven genes were expressed positively, i.e., above the expression found in normal tissue, indicating that the lung responds with a positive expression profile when a remote organ is subjected ischemia and reperfusion. Based on our protocol, the gene expression levels greater than three-fold above the threshold of the algorithm comparisons between normal tissue (CG) and the tissue subjected to ischemic stimulus are significant. Thus, two genes were up-regulated with over expression: Gpx4(+4.10) and Lpo(+3.51).

The Gpx4 gene encodes a protein that is part of a family of enzymes related to the glutathione peroxidases. Glutathione is a tripeptide synthesized in the body from L-cysteine, glutamic acid, and glycine. The sulfhydryl radical (thiol) cysteine has the role of proton donor and is responsible for its biological activity. The availability of these amino acids is a limiting factor in the synthesis of this enzyme by the cells largely because cysteine is poorly available in the diet[16]. Glutathione is found almost exclusively in its reduced form (glutathione reductase), which ischemically activated by oxidative stress, i.e., by upsetting the balance between reactive oxygen species and cellular and humoral natural antioxidant defenses[17,18].

Glutathione peroxidases have the important biological function of protecting the organism from the harmful effects of oxidative stress. They are present in almost all cells of the animal kingdom, involved in the reduction of lipid hydroperoxides to the corresponding alcohols, and in the oxidation of free hydrogen peroxide (H_2O_2) to water. Thus, glutathione peroxidases play an active role in removing reactive oxygen species (ROS) inside cells[16-20]. Reduced glutathione is present in high concentrations in bronchial lavage fluid and lung alveoli, thus protecting them against oxidative damage. The importance of glutathione peroxidases has been confirmed in studies in which its depletion was correlated with an increased risk of pulmonary disease[21]. One of the proposals found in the literature to lower or abolish the effects of oxidative stress is the exogenous addition of glutathione or its precursor, n-acetylcysteine[22,23]. A higher dose of such substances, even if controversial, may be beneficial because as the dose and duration of the treatment seems to be of value for reducing the damage involved in the ischemic tissue.

The GPx4 protein, which is found in the nucleus, the mitochondria, the cytosol, and the cell membrane, has a preference for lipid hydroperoxides that are present in nearly every mammalian cell. It is unique among the glutathione peroxidases because it uses phospholipid hydroperoxide as its substrate[24] and reacts with a wide variety of other hydroperoxides, including cholesterol derivatives and thymine[25]. The protein GPx4, together with d-alpha-tocopherol, prevents oxidative damage of the membranes. In addition to antioxidant activity, GPx4 has been implicated to be a structural component in the maturation of sperm[26], a regulatory protein in programmed cell death[27], and involved in eicosanoids biosynthesis[28].

Based on what is known about the activity of the GPx4 protein, the over expression of the Gpx4 gene identified in this research suggests that the genetic mechanism responsible for the antioxidant defense in lung tissue responded meaningfully to the aggressive stimulus triggered by remote oxidative stress from intestinal ischemia and reperfusion injury. This finding opens the possibility of trying to identify which pathway, humoral or cellular, is primarily responsible for the specific gene expression. It is also of importance to determine what will happen to gene expression patterns produced by oxidative stress over a longer observation period. Will there be a corresponding production of the encoded protein that could be identified by Immunohistochemistry or proteomics? The merit of this research is the identification of multiple antioxidant genes, there by guiding future research.

The other gene determined to be up-regulated with over expression, was the Lpo gene belonging to the family of peroxidases, which are characterized as enzyme catalysts. For many of these enzymes, the optimal substrate is hydrogen peroxide; however, others use mostly active substrates, such as organic hydroperoxides (lipid peroxides). The peroxidase may contain a heme cofactor in their active sites (redox-active selenocysteine or cysteine residues). Although the exact mechanisms for peroxidases have not been elucidated, peroxidases are known to play a role in the modulation of the oxidative stress[29].

LPO oxidoreductase is an enzyme present in the salivary glands, mammary glands, and other mucous membranes and acts as a natural antimicrobial agent[30-32]. It is associated with the epithelial cells involved in intestinal absorption and secretion. LPO is secreted in milk and plays an important role in protecting both the mammary gland during lactation and the intestinal tract of infants against pathogenic microorganisms[30]. Other function shave been reported, such as promoting cell growth[33] and antitumor activity[34]. In the lung, LPO is present in the submucosal glands of the trachea, bronchi, and the normal respiratory secretions, where it exerts its enzymatic activity[35]. Although lactoperoxidase does not harm DNA, under certain circumstances it can contribute to oxidative stress[36]. The over expression of Lpo in our model can be associated with the presence of excessive amounts of ROS, especially hydrogen peroxide and organic hydroperoxides. This may lead to oxidative stress in the lung tissue resulting from intestinal ischemia and reperfusion, suggesting a possible protective action to the deleterious effects of ROS[29].

Ischemia and reperfusion in the intestine was able to stimulate lung cell genomic machinery. In the model used, as early as the first hour of reperfusion, one hour after clamping, the intestinal vascular displayed a positive expression profile for 67 genes out of a total of 84. The time evolution of the genomic response is unknown and opens the possibility for assessing more time points to establish a time line of gene expression events.

In a recent publication evaluating gene expression in the intestine[14] using the same technique performed in our research, 37 genes were expressed greater than three-fold, which represents a significant response to stimuli triggered by ROS and antioxidant defense in the studied tissue. In the results reported here, however, only two genes (Gpx4 and Lpo) were expressed above three fold the threshold. This seems reasonable because ischemia and reperfusion in the intestine is expected to be much more severe than in distant tissues. Moreover, in lung tissue, there is a peculiarity; oxygen is present in the alveoli in two manners, perfusion and ventilation[37]. In this condition, the alveolar oxygen helps to maintain aerobic metabolism by delaying hypoxia, and this mechanism protects and slows oxidative stress in lung tissue, which decreases the need for antioxidant protein production compared to other tissue, such as in the bowel.

The objective of this research was to identify and outline the oxidative stress gene expression profile in lung tissue after distant organs were subjected to ischemia and reperfusion. The genes identified here as being up-regulated need further validation. For example, the results may be confirmed using proteomics. Indeed, the identification and quantification of protein in the tissue may provide information as to whether the gene expression functioned as a coding stimulus or whether protein expression is related to the signaling or modulation of other metabolic pathways of the oxidative stress response.

This study is promising because the knowledge generated from gene expression analyses could result in the development of procedures for monitoring the progression and treatment of multiple organ failure and, specifically, for monitoring the involvement of lung tissue in this phenomenon. As a starting point for further studies, the screen performed in the present study allowed us to identify two genes that were significantly responsive to oxidative stress.

Conclusion

An intestinal ischemia/reperfusion injury promoted the positive expression of oxidative genes in a remote organ (i.e., lung), suggesting that activated signaling pathways are implicated in cell survival and the maintenance of genome integrity.

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Correspondence:
Adauto Tsutomu Ikejiri
Rua João Vicente Ferreira, 1789
79824-030 Dourados – MS Brasil
Tel.: (55 67)3421-7171
adalto.ike@uol.com.br

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