Time course of COX-1 and COX-2 expression during ischemia-reperfusion in rat skeletal muscle

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Dupouy, V. M., P. J. Ferre, E. Uro-Coste, and H. P. Lefebvre. Time course of COX-1 and COX-2 expression during ischemia-reperfusion in rat skeletal muscle. J Appl Physiol 100: 233–239, 2006; doi:10.1152/japplphysiol.00673.2004.—The aim of this study was to assess cyclooxygenase (COX)-1 and COX-2 expression in skeletal muscle after an ischemia-reperfusion (I/R). Male Sprague-Dawley rats were subjected to unilateral hindlimb ischemia for 2 h and then euthanized after 0, 1, 2, 4, 6, 10, 24, and 72 h of reperfusion. The COX protein and mRNA were assessed in control and injured gastrocnemius muscle. Muscle damage was indirectly determined by plasma creatine kinase activity and edema by weighing wet muscle. Creatine kinase activity in plasma increased as early as 1 h after reperfusion and returned to control levels by 72 h of reperfusion. Edema was observed at 6 and 10 h of reperfusion, but histological investigations showed an absence of tissular inflammatory cell infiltration. COX-1 mRNA was expressed in control muscle and was increased at 72 h of reperfusion, but the levels of associated COX-1 protein detected in control and injured gastrocnemius muscle were similar. COX-2 mRNA was not, or only slightly, detectable in control muscle and after I/R. In contrast, I/R induced major overexpression of COX-2 immunoreactivity at 6 and 10 h of reperfusion with a maximum at 10 h, whereas COX-2 protein was undetectable in control muscle. In conclusion, hindlimb I/R induced a large overexpression of COX-2 but not COX-1 protein between 6 and 10 h after injury. These results suggest a role for COX-2 enzyme in such pathophysiological conditions of the skeletal muscle.

cyclooxygenase; skeletal muscle injury; inflammation

THE CYCLOOXYGENASE (COX) isoforms, COX-1 and COX-2, catalyze the initial step in the metabolism of arachidonic acid to the intermediate prostaglandin H2, which is converted to various prostaglandins and thromboxanes by cell-specific enzymes. COX-1 is often constitutively present, and COX-2 is not expressed or at very low levels in most normal tissues but may be rapidly upregulated in pathological conditions. Expression of COX isoforms differs according to physiological and pathological conditions. For example, under physiological conditions in rats, COX-1 mRNA is expressed in the liver, kidney, and lung but not in the heart, whereas COX-2 mRNA is expressed in the kidney, heart, and lung but not in the liver (36). Ischemia-reperfusion (I/R) is a common physiopathological process, and I/R-induced changes in COX-1/COX-2 expression have been reported in various organs and tissues, such as brain (38), stomach (28), myocardium (40), kidney (41), and more recently retina (25).

In contrast, COX expression in skeletal muscle has been poorly documented. Acute ischemia followed by reperfusion provides a relevant model in musculoskeletal pathology as it is a common complication of trauma, hemorrhage, vascular stenosis, and thromboembolic events. Although surgical treatment generally allows rapid recovery of skeletal muscle, the restoration of blood flow following ischemia may also induce severe injury characterized by edema, muscle cell lesions, alteration of local blood flow, and loss of muscle function. The mechanisms by which reperfusion of ischemia muscle produce injury remain uncertain. Cytotoxic events initiated at the onset of reperfusion involve 1) the formation of reactive oxygen metabolites leading to microvascular and muscle dysfunction, 2) protease activity, 3) vasoactive molecules, and mediators of protein turnover in skeletal muscle, and because arachidonic metabolism is a source of cytotoxic oxygen metabolites (22, 46). Second, dexamethasone decreases in vitro COX activity in skeletal muscle (6) and improves the survival rate of ischemic-reperfused muscle (30) likely due to a combination of actions, such as the inhibition of production of several inflammatory mediators.

However, no study to our knowledge has investigated the basal levels of COX-1 and COX-2 mRNA and protein in healthy and I/R conditions, despite the fact that the COX system should be a relevant candidate for explaining some pathophysiological features of I/R in skeletal muscle.

In the present study, we addressed the question of the constitutive expression of COX and whether COX expression is associated with the pathophysiology of I/R in muscle. We assessed COX-1 and COX-2 mRNA and protein expression in rat skeletal muscle under normal conditions and after I/R.

MATERIALS AND METHODS

Animal model. Forty male Sprague-Dawley rats (HarlanFrance, Gannat, France), weighing 262–316 g, were divided into eight groups of five rats. They were housed in plastic cages and allowed free access to standard pellet chow and water. All experiments were performed according to the Guide For Care and Use of Laboratory Animals (23).
I/R model and sampling. The model selected in the present study was the rat hindlimb tourniquet ischemia model (42). Briefly, rats were anesthetized (chloralose 0.2 g/kg and urethane 2 g/kg ip), the left hindlimb was shaved, and a size 13-mm rubber band was placed around the thigh as high up as possible. Ischemia was maintained for 2 h followed by various times of reperfusion until euthanasia of the group, after 0, 1, 2, 4, 6, 10, 24, or 72 h. At the end of the reperfusion period, blood was sampled from the abdominal aorta and the rats were euthanized by intracardiac pentobarbital injection. After 4, 6, 10, 24, and 72 h of reperfusion, isoflurane anesthesia was performed just before euthanasia to allow blood sampling. Left (injured) and right (control) gastrocnemius muscles were then harvested. Immediately after weighing, standardized transversal sections of the superficial and deep gastrocnemius muscles were performed by the same trained operator.

Assessment of muscle edema and damage. The I/R and contralateral control gastrocnemius muscles were weighed. The relative increase (%) in the weight of injured muscle vs. contralateral was calculated. Muscle damage was assessed from the plasma creatine kinase (CK) activity, according to the recommendations of the International Federation of Clinical Chemistry (20). Briefly, an enzymatic kit was used (Enzyline CK NAC optimisé unitaire, BioMérieux, Craponne, France). The activity of CK was determined from the increase in optical density by spectrophotometry and ethidium bromide staining.

Single-strand cDNAs were synthesized from total RNA using oligo(dT) priming with SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Pontoise, France). α-32P-labeled dCTP (0.5 µCi; Amersham Pharmacia Biotech) was added to monitor the transcription. For each sample, a reaction without transcriptase was run as a control for DNA contamination. Negative (water replacing RNA in the RT reaction) and positive controls (RNA from muscle with detectable COX-2 mRNA in the RT-PCR reaction) were included in each RT run. After 1 h of incubation, the RNA was hydrolyzed and cDNA was purified by G50 chromatography and quantified by measuring the radioactivity incorporated. cDNA was further precipitated with ethanol-NaCl and resuspended in sterile water to obtain a concentration of 1 ng cDNA/µl.

PCR reactions were performed in a total volume of 25 µl. Five microliters of diluted cDNA (i.e., 100, 50, and 25 pg/µl) were amplified in a 20-µl PCR mixture containing 2.5 µl of 10× PCR buffer, 2.5 µl of 2 mM dNTP cocktail, 2.5 µl of 15 mM MgCl2, 1 U of Taq DNA polymerase (Invitrogen Diagnostics), and 1 µl of the final concentration of both sense and antisense primers. Negative (water replacing cDNA in the PCR reaction) and positive controls (cDNA from muscle with detectable COX-2 mRNA in the PCR reaction) were included in each PCR run. Primer selection was based on previously published rat COX-1 (13), COX-2 (26), and glycyrrhetinic acid 3-phosphate dehydrogenase (GAPDH) (44) sequences in the GenBank database. A 435-bp COX-1 fragment was amplified using the following primers: sense, 5’-GCC GGA TTG GGT GGA GTT CTT G3’ and antisense, 5’-AGG GCC AGG CCT TGG TGT TGG C3’.

A 388-bp GAPDH fragment was amplified using the following primers: sense, 5’-TAT CTC CCA GCC GCC CTG CTG GTG-3’ and antisense, 5’-ACT TGT GAT GAT GGT GGT CTG CTG-3’. The PCR amplification was performed on a MJ Research PTC 200 thermocycler as follows: initial denaturation at 94°C for 5 min followed by 19–34 cycles of amplification (1 min at 94°C, 30 s at the specific hybridization temperature of each primer pair, 1 min at 72°C). We performed 19 PCR cycles with an annealing temperature at 60°C for GAPDH, 31 cycles at 64°C for COX-1, and 34 cycles at 70°C for COX-2. The number of cycles was selected so as to fall within the exponential phase of the amplification reaction for the cDNA dilution assessed. PCR reaction products were resolved by electrophoresis through a 1% agarose gel. The amplified DNA fragments were detected by ethidium bromide staining, and the band density was determined with Image Quant Software Bio-1D V99 (Vilber Lourmat, Marne-La-Vallée, France). The COX values were normalized against GAPDH for analysis.

Statistics. CK, muscle weight, COX-1 mRNA, COX-1 protein, and COX-2 protein data are presented as means (SD). Statistical analysis was performed after log transformation to homogenized variance when appropriate (plasma CK activity, COX-2 protein). Data were subjected to an analysis of variance, and significant differences were assessed by Dunnet’s test with P < 0.05 for comparison with the control value (2-h ischemia and no reperfusion). A power analysis was also performed to explore the relationship between our sample size (5 animals per group) and the probability of achieving statistical significance. Groups showing a significant difference from the control value
RESULTS

Pathological findings. Two hours of hindlimb I/R induced edema with a maximum value observed after 10 h of reperfusion [muscle weight increased by 25.6% (SD 9.8) vs. control] (Fig. 1).

Plasma CK activity was 608 U/l (SD 413) after 2 h of ischemia without reperfusion, then increased significantly (Dunnett’s test, $P < 0.05$) to plateau between 1 and 24 h of reperfusion (maximum value: 3,530 U/l (SD 3,240) and returned to the control level by 72 h of reperfusion [77 U/l (SD 15)] (Fig. 2).

In almost all samples, microscopic examination of gastrocnemius muscle did not show any histological difference (necrosis, inflammatory cells) between the ischemic and the control muscle at any time after reperfusion (Fig. 3). The only exception was the observation of foci of necrotic cells in two of the five rats killed 10 h after reperfusion.

COX protein and mRNA in skeletal muscle. Expression of COX-1 mRNA was observed in the control and I/R muscles with consistent overexpression after 72 h of reperfusion (Dunnett’s test, $P < 0.005$) (Fig. 4, A and C). Up to 24 h after reperfusion, the power analysis indicated that the observed difference would have been statistically significant for a sample size of at least 52 rats per group because of a large between-subjects variability or the absence of (or minimal) observed differences.

The expression of COX-2 mRNA was undetectable or only faintly detectable in control muscle and after I/R. At 1, 10, and 72 h of reperfusion, a mild signal was only detectable after I/R in three animals per group. This signal was only slightly distinguishable from noise. Thus no modification of COX-2 mRNA expression after I/R was evidenced.

COX-1 protein immunoreactivity at 70 kDa was revealed in gastrocnemius muscle under basal conditions, but no detectable changes occurred after ischemia of the hindlimb and 0 to 72 h of reperfusion (Dunnett’s test, $P > 0.05$) (Fig. 4, B and C). A power analysis indicated that the observed differences would have been statistically significant for a sample size of 10 rats per group after 4, 6, 72 h of reperfusion, but such differences were very small and probably without biological meaning [COX-1 protein as fold of the contralateral control was 1.10 (SD 0.25) without reperfusion, 1.34 (SD 0.42), 0.86 (SD 0.26), and 1.33 (SD 0.42) after 4, 6, and 72 h of reperfusion, respectively].

No COX-2 protein immunoreactivity could be detected in control muscle. However, ischemia for 2 h followed by 6 or 10 h of reperfusion triggered a major overexpression of COX-2 67-kDa protein with a maximum at 10 h of reperfusion (Dunnett’s test, $P < 0.005$). No COX-2 protein was statistically detected before and after this 10-h sampling time (Dunnett’s test, $P > 0.05$) (Fig. 5).

DISCUSSION

This study is the first to assess COX-1 and COX-2 mRNA and protein in skeletal muscle in control and I/R conditions. Skeletal muscle necrosis after I/R injury is a major issue in plastic and reconstructive surgery (27) and is a significant cause of morbidity after traumatic injury (1). The tourniquet hindlimb model is considered to appropriately mimic these clinical situations. We selected the gastrocnemius muscle for our experiments. One potential issue is that gastrocnemius muscle contains both white and red muscle fibers, which can be difficult to distinguish from each other. However, the sampling procedure was normalized and performed by the same trained investigator using transversal sections, and it is reasonable to assume that similar proportions of all fiber types were present in each individual sample. The white fibers are mainly located in the superficial portion of the gastrocnemius, whereas the red fibers are located deeper in the muscle. The level of irreversible muscle damage is related to the duration of ischemia but not to reperfusion. In a preliminary experiment (data not shown), we found that a 4-h period of ischemia induced high mortality and morbidity in rats and prevented experiments up to 3 days after the end of ischemia. A follow-up of 3 days was indeed required to accurately document the time development of the muscle damage, inflammation process, and potential concomitant changes in COX-1/COX-2 expression. A 2-h period of ischemia induced damage that was reversible and allowed adequate...
investigation, as previously shown in other studies of I/R injury and potential therapeutic strategies (30, 50).

In our study, 2 h of ischemia induced muscle damage and edema as evidenced by an increase of both plasma CK activity and muscle weight. Edema was maximal after 10 h of reperfusion. Plasma CK activity is considered as the best marker of muscle damage (34). A dramatic increase in plasma CK activity was observed after as little as 1 h of reperfusion (threefold vs. no reperfusion) and then plateaued through 24 h of reperfusion (sixfold vs. no reperfusion). This CK response pattern is probably due to an immediate CK release from muscle cells adjacent to the vascular system and then to arrival in the systemic circulation of bulk CK with the lymph. Microscopic examination of injured muscles revealed no changes, except in two rats (foci of necrotic muscle cells) after 10 h of reperfusion. However, the increase in plasma CK activity after 1 h of reperfusion indicated a profound alteration of muscle cell permeability early in the time course of lesion development. Ultrastructural damage of the skeletal muscle is normally observed after 2 h of ischemia and 1 h of reperfusion that coincides with the process of membrane lipid peroxidation by excessive formation of oxygen-free radicals (35). In the present experiment, microscopic examination did not show any extracellular leukocyte infiltration after several hours of reperfusion, although edema was evidenced at 6 and 10 h of reperfusion by the increase in muscle weight. Edema formation is considered important in the development of muscle injury in skeletal muscle (18). The precise mechanism of such edema formation has not been entirely elucidated, but it can be hypothesized that the tourniquet model creates a state of tissue anoxia/hyperoxia that produces an increase in endothelial vascular permeability resulting in a loss of fluid and protein into the tissue (exudate) contributing to edema. Activation of resident phagocytes (14) or complement (31, 32) may be involved in this reaction of the microcirculation. It was reported by others that, after 2 h of ischemia, a full inflammatory response was initiated in I/R hindlimb with an increase of leukocyte rolling as early as 30 min that peaked at 2 h. The rolling then stopped, and adhesion occurred before possible transendothelial migration. Leukocyte adhesion increased up to 4 h after reperfusion and was associated with muscle edema but with no evidence of infiltration (8). In our study, the occurrence of muscle damage and edema is in agreement with other reports on similar models, whereas reports of the presence or absence of leukocyte transmigration differ greatly according to authors (8, 19). It is worth noting that, if any necrosis of the muscle cells occurred, an infiltration of macrophages was observed to phagocytize such cells between 24 and 72 h after reperfusion and thus initiate skeletal muscle regeneration (24). Finally, it is likely that an inflammatory response was initiated under our I/R conditions but would have been limited to the early events (edema without leukocyte migration) due to the absence or minimal formation of any necrotic tissue.

COX-1 mRNA and its associated protein were detectable in control conditions. This is the first evidence of COX-1 constitutive expression in skeletal muscle. This constitutive expression could be related to some housekeeping functions of COX-1 as observed in other tissues (33). COX-1 might also be involved in regulation of muscle microcirculation as COX products demonstrate vasoactive properties on skeletal muscle arterioles and venules (11, 15). Furthermore, COX might regulate basal protein turnover as prostaglandins have been detected in skeletal muscle, regulate protein synthesis and degradation in muscle (46), and are involved in various stages of myogenesis such as myoblast proliferation (49), differentiation (39), and fusion (21).

After I/R, a late COX-1 mRNA overexpression was observed 72 h after reperfusion but without a corresponding increase of COX-1 protein. The induction of COX-1 mRNA levels, without a concomitant increase in COX-1 protein, has already been reported in mouse NIH 3T3 cells (9) and has been explained in terms of a posttranslational regulation of COX-1.
COX-2 expression was undetectable or low, with a rapid and transient overexpression of mRNA and/or protein of COX-2 after I/R in stomach (28), retina (25), kidney (41), and heart (40). An absence of mRNA overexpression coupled with an overexpression of the protein has been previously observed for COX-2 in rat bladder inflammation (48) and skeletal muscle during the early stages of muscle regeneration (2). In vitro studies demonstrated that the COX-2 control elements of the 3′-untranslated region mediate the posttranscriptional regulation by changing mRNA stability and translational efficiency (10, 16). The lack of apparent COX-2 mRNA response to I/R may be explained by extreme instability of the mRNA, as previously demonstrated in rat skeletal muscle (half-life of 5–10 min) (12). Alternative mechanisms other than mRNA stability might explain mRNA and protein dissociation, such as a more efficient translation and/or a relatively low turnover of protein that may induce an observable increase in COX-2 protein, while COX-2 mRNA remains not or poorly detectable. Furthermore, mRNA levels may change considerably between individuals showing similar levels of protein (7). Our results, together with those in the literature, suggest that the skeletal muscle I/R triggers a transient upregulation of the COX-2 protein, suggesting a time-limited role of COX-2 in pathophysiological I/R in skeletal muscle. As the time course of COX-2 overexpression was simultaneous to that of the edema formation, it can be tentatively suggested that COX-2 plays a role in this formation, since eicosanoids cause vasodilation due to activation of the resident phagocytes (14) and prolong edema. It is clear that the power of our experimental design with five rats per group was relatively low so that only large (and thus relevant) differences were a priori detectable. A power analysis (results not shown) indicated that our conclusions, obtained

insufficient changes in COX-1 protein regarding its constitutive expression, or a possible induction of aberrantly spliced COX-1 mRNA, as previously suggested in other models (29). The fact that COX-1 protein expression remained at the basal level during the reperfusion injury phase does not rule out a role of COX-1 in the pathophysiology of I/R. Indeed, it has been demonstrated that basal COX-1 expression, despite any upregulation of COX-2, is sufficient to have a crucial role in gastric mucosa defense (3, 4, 45).

In basal conditions, the level of COX-2 mRNA was undetectable or faintly detectable, but no protein was evidenced. I/R caused no change in COX-2 mRNA expression. In contrast, I/R triggered a large overexpression of COX-2 protein 6 and 10 h after reperfusion, i.e., during edema development. These results are in agreement with the COX-2 expression profile previously described in other tissues. Under basal conditions,

Fig. 4. Effect of 2 h of hindlimb ischemia and 0–72 h of reperfusion on cyclooxygenase (COX)-1 mRNA and protein expression in gastrocnemius muscle. A: representative RT-PCR profile of COX-1 and GAPDH mRNA expression from a single cDNA dilution titer at each time of muscle collection. RT-PCR products were separated in 1% agarose gel and stained with ethidium bromide. B: COX-1 immunoreactivity in test (+) and contralateral control (−) muscles. Representative Western blots of muscle extracts at each time of tissue collection are shown. Solubilized microsomes (100 µg/lane) were probed with anti-COX-1 1/500. The signal was revealed by enhanced chemiluminescence. C: COX-1 mRNA and protein were quantified by densitometry. Relative COX-1/GAPDH mRNA abundance was determined by semi-quantitative analysis. Data are expressed as fold increase of COX-1 mRNA in ischemia-reperfusion vs. control contralateral muscle [means (SD)]. Data were subjected to an analysis of variance followed by a Dunnett’s test for comparison with the control value (2 h of ischemia and no reperfusion). ***P < 0.005.

Fig. 5. Effect of 2 h of hindlimb ischemia and 0–72 h of reperfusion on COX-2 protein expression in gastrocnemius muscle. A: COX-2 immunoreactivity in test (+) and contralateral control (−) muscles. Representative Western blots of muscle extracts at each time of tissue collection are shown. Solubilized microsomes (100 µg/lane) were probed with anti-COX-2 1/1,000. The signal was revealed by enhanced chemiluminescence. B: COX-2 protein was quantified by densitometry. Data are expressed as fold increase of COX-2 protein in ischemia-reperfusion vs. control contralateral muscle [means (SD)]. Data were subjected to an analysis of variance (after log transformation) of the data followed by a Dunnett’s test for comparison with the control value (2 h of ischemia and no reperfusion). *P < 0.05; ***P < 0.005.
with five rats per group, would not have been essentially modified by doubling the number of animals per group.

The beneficial or detrimental effects of COX activity on I/R-induced muscle injury remain to be elucidated. Dexamethasone reduces loss of muscle viability after I/R injury (30) and decreases COX activity in skeletal muscle (6). However, these data are not necessarily predictive of a putative ability of COX-2 inhibitor to prevent muscle I/R damage. Selective COX inhibition in injury models of brain (22) and heart (37) could produce beneficial, ineffective, or detrimental effects.

Several limitations of the present work should, however, be emphasized. First, the mechanisms underlying the alteration of COX expression remain unidentified; second, the explanation of the discrepancy between COX mRNA and protein expression is rather speculative, and there are no data at our disposal to support or negate the proposed explanation; last, because of the great variability of COX expression, the number of animals per group in our trial could not provide definitive answers, and a very large number would be required for this purpose.

In conclusion, our results are in agreement with those reported in various tissue-injury models, showing a constitutive basal expression of COX-1 and an upregulation of COX-2 during the acute injury phase. These results may be useful for understanding COX in the development stages of muscle ischemia injury and should encourage further studies, especially the pharmacological blocking of COX-1 and COX-2 with selective NSAIDs.

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REFERENCES


