Oxidative stress precedes skeletal muscle mitochondrial dysfunction during experimental aortic cross-clamping but is not associated with early lung, heart, brain, liver, or kidney mitochondrial impairment

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Objective: **Lower limb ischemia-reperfusion results in skeletal muscle mitochondrial alterations, production of reactive oxygen species (ROS), and remote organ impairments that are largely involved in patient prognosis. However, whether ischemia without reperfusion increases ROS production and precedes mitochondrial alteration and whether mitochondrial dysfunction occurs early in remote organs is unknown. This study determined muscle mitochondrial function and ROS production after ischemia alone, or followed by two periods of reperfusion, and investigated heart, lung, liver, kidney, and brain mitochondrial functions after lower limb ischemia-reperfusion.**

Methods: **Wistar rats were randomized into four groups: sham (aortic exposure but no ischemia, n = 9), I3 (ischemia alone induced by aortic cross-clamping for 3 hours, n = 9), I3R10′ and I3R2 (aortic cross-clamping, followed by reperfusion for 10 minutes [n = 8] or 2 hours [n = 9]). Blood lactate, alanine aminotransferase, aspartate aminotransferase, and creatinine were measured. Mitochondrial respiratory chain complexes I, II, III, and IV activities and mitochondrial coupling (acceptor control ratio) were analyzed using a Clark oxygen electrode in skeletal muscle, lung, heart, brain, liver, and kidney. ROS production was determined using dihydroethidium staining in muscle, heart, liver, and kidney. Inflammation was also investigated in remote organs (heart, liver, and kidney) using monocyte-macrophage-2 antibody staining.**

Results: **Lactate level increased after ischemia in all groups. In muscle, ROS increased significantly after ischemia alone (+324% ± 66%; $P = .038$), normalized after 10 minutes of reperfusion, and increased again at 2 hours of reperfusion (+349.2 ± 67%; $P = .024$). Interestingly, mitochondrial function was unaffected by ischemia alone or followed by 10 minutes of reperfusion, but maximal mitochondrial oxidative capacity (6.10 ± 0.51 vs 4.24 ± 0.36 μmol/min/g, −30%; $P < .05$) and mitochondrial coupling decreased after 2 hours of reperfusion (1.93 ± 0.17 vs 1.33 ± 0.07, −45%; $P < .01$), in sham and I3R2 rats, respectively. Despite increased serum aspartate aminotransferase (×13; $P < .0001$), alanine aminotransferase (×6; $P = .0019$), and creatinine (×3; $P = .0004$), remote organs did not show mitochondrial alteration, inflammation, or ROS production enhancement after 2 hours of reperfusion.**

Conclusions: **Oxidative stress precedes skeletal muscle mitochondrial dysfunction during lower limb ischemia. Such a kinetic explains the efficacy of ischemic preconditioning and supports that therapy should be conducted even during ongoing ischemia, suggesting that ischemic preconditioning might be a successful approach. (J Vasc Surg 2014;60:1043-51.)**

Clinical Relevance: **Aortic cross-clamping increases reactive oxygen species (ROS) and impairs skeletal muscle and remote organs, which is involved in patient prognosis. However, the temporal relationship between ROS production and mitochondrial dysfunction during lower limb ischemia reperfusion is unknown. This study demonstrates for the first time that ROS production occurs during ischemia alone, without reperfusion, and precedes skeletal muscle mitochondrial impairments. Although involved in multiorgan failure, lung, heart, brain, liver, and kidney mitochondria are not affected early. These results support a need for muscle protection even during lower limb ischemia and that ischemic preconditioning (conditioning performed during ongoing ischemia) might be a successful approach.**

Aortic cross-clamping is often performed in the setting of vascular surgery, such as abdominal aortic aneurysm repair and might be associated with significant local and remote complications. Rhabdomyolysis is not needed to complicate outcomes, and subtle intraoperative skeletal muscle ischemia also contributes to remote organ

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injuries, contributing to immediate and to long-term morbidities.[1-4]

Key factors involved in the deleterious effects of lower limb ischemia-reperfusion (IR) are increased oxidative stress and impaired mitochondrial functions.[5-7] Increased reactive oxygen species (ROS) production is known to alter cell lipids, proteins, and DNA and to mediate mitochondrial dysfunction in skeletal muscles. In turn, impaired mitochondria enhance their production of ROS in a vicious cycle in which mitochondria are both the target and origin of increased oxidative stress.[8]

A better knowledge on the precise temporal relationship between mitochondrial dysfunction and increased ROS production is mandatory to improve muscle protection. The precise kinetic of alteration of the respiratory chain and free-radical production is not known during lower limb IR. Production of ROS occurs in response to ischemia at the time of reperfusion,[9] but whether ROS might arise early during ischemia without reperfusion in skeletal muscle remains an unresolved issue. Similarly, mitochondrial dysfunction occurs after ischemia of >5 hours or after 3 hours of ischemia, followed by 2 hours of reperfusion,[7,9,10] but whether mitochondrial dysfunction occurs during shorter-term ischemia without reperfusion is not known.

Another interesting issue is remote organ impairments. Although remote organ impairment participates greatly in patient prognosis,[1-4] few experiments have investigated potential remote organ alterations at the mitochondrial level after lower limb IR. In view of its clinical importance, mitochondrial dysfunction, often leading to organ dysfunction and ultimately to multiorgan failure syndrome,[11] and main organ mitochondrial function after aortic cross-clamping and reperfusion deserve to be studied to develop and compare the effectiveness of clinical strategies to reduce cardiovascular and other perioperative complications after vascular interventions.[12]

The first objective of our study was therefore to determine simultaneously the kinetics of ROS production and mitochondrial respiration changes in four experimental settings, challenging the hypothesis that ROS production might occur in skeletal muscle during ischemia alone and might precede mitochondrial dysfunction. Further, we investigated whether aortic cross-clamping impairs five main organs involved in multiorgan failure—lung, heart, brain, liver, and kidney—through a combination of biologic, histologic, and mitochondrial function analysis.

METHODS

The investigations in this study were performed in accordance with the Helsinki Accords for Humane Treatment of Animals During Experimentation. We obtained approval from the Comité régional d'éthique en matière d'expérimentation animale (CREMEAS), the ethics committee on animal research.

Study animals. The study was performed in adult male Wistar rats (250 to 350 g). The rats were housed in a thermoneutral environment of 22° ± 2°C on a 12-hour day/night cycle and were provided food and water ad libitum.

Preoperative management. Rats were placed in a hermetic cage for induction of anesthesia and ventilated with a mixture of 4% isoflurane (Centre Spécialités Pharmaceutiques, Cournon, France) and oxygen. After induction, rats were placed on heating blankets (Equipement Vétérinaire Minerve SA, Esternay, France), with a preselected 35°C temperature maintained during the entire procedure. Spontaneous ventilation was allowed through an oxygen-delivering mask, with concentrations of isoflurane adapted to the surgical phases. A rectal probe was inserted for continuous temperature monitoring, and three electrodes were placed for electrocardiographic monitoring.

Experimental design and surgical procedure. Rats were divided into four groups. The sham group (n = 9) had no ischemia but underwent anesthesia, and a midline laparotomy was performed. The posterior peritoneum was cut to expose the abdominal aorta and the inferior vena cava. The infrarenal abdominal aorta was dissected and liberated from adjacent adhesions in all animals. The ischemia-alone group (I3, n = 9) underwent 3 hours of ischemia induced by infrarenal aortic occlusion, and arterial collaterals located between the renal arteries and the aortic bifurcation were coagulated using a Geiger Thermal Cautery Unit (Geiger Medical Technologies, Council Bluffs, Iowa) and cut.

The two IR groups also underwent 3 hours of ischemia, followed by reperfusion for 10 minutes (I3R10′, n = 8) or 2 hours (I3R2, n = 9). The aorta was temporally occluded using a DeBakey Cross-Action Bulldog Clamp, as reported in previous experiments.[9,10,13] After aortic clamping, the abdomen was tightly closed to avoid dehydration. Ischemia was clinically characterized by cyanosis and lack of arterial pulse distal to the clamp and was also ascertained by capillary lactate measurement on the tail (Lactate Pro; Arkray SAS, Paris France).

At the end of the experiment, the right gastrocnemius muscle was dissected for immediate mitochondrial function analysis. Part of the muscle was also cryopreserved for ROS production determination.

Detection of ROS. To detect the presence of ROS in skeletal muscle, heart, kidney, and liver, serial 10-μm sections were cut on a cryostat microtome, mounted onto glass slides, and incubated with 2.5 μmol/L dihydroethidium (DHE)[14,15] (Supplementary Methods, online only).

Immunohistochemical procedure. To detect inflammation in heart, kidney, and liver in the sham and I3R2 groups at the end of experiment, we used monocyte-macrophage-2 antibody (Millipore, Billerica, Mass) on 10-mm-thick serial sections of tisuse[10] (Supplementary Methods, online only).

Permeabilization of muscle and heart fibers and isolation of mitochondria in lung, heart, brain, liver, and kidney. Mitochondrial respiration was studied in saponin-skinned fibers, as previously described.[16,17] Lung, heart, brain, liver, and kidney mitochondria were isolated on ice, homogenized, and centrifuged. Aliquots were then

removed for protein measurement by the Bradford method (Biomate 3; Thermo Fisher Scientific Inc, Waltham, Mass) (Supplementary Methods, online only).

Study of activities of muscle mitochondrial respiratory chain complexes and mitochondrial coupling. Measuring oxygen consumption in skinned fibers is a unique technique to determine the oxidative capacity of the skeletal muscle in its cellular environment, and mitochondrial oxygen consumption is measured polarographically by using a Clark-type electrode (Strathkelvin Instruments, Glasgow, Scotland) and expressed in micromoles of oxygen consumed per minute reported on the dry weight of muscle sample (μmol/min/g). The relative contribution of the respiratory chain complexes I, II, III, and IV to the global mitochondrial respiratory rate can be studied using different substrates.

First, basal respiration (V0) with glutamate and malate as substrates was analyzed. Then, maximal fibers respiration (Vmax) was recorded after adding adenosine diphosphate (ADP). In this case, electron flow went through complexes I, III, and IV because of the presence of glutamate (5 mmol/L) and malate (2 mmol/L).

Thereafter, complex I was blocked with amobarbital (0.02 mmol/L), and complex II was stimulated with succinate (25 mmol/L). Mitochondrial respiration in these conditions allowed the investigation of complexes II, III, and IV activities (Vsucc).

Finally, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD, 0.5 mmol/L) and ascorbate (0.5 mmol/L) were added as an artificial electron donor to complex IV. Complex IV activity was then determined as an isolated step of the respiratory chain (Vtmpd).

Mitochondrial coupling (coupling of phosphorylation to oxidation) was determined by calculating the acceptor control ratio (ACR), the ratio between ADP-stimulated respiration (Vmax) over basal respiration (V0)[9,10,13] (Supplementary Fig 1, online only).

Serum alanine aminotransferase, aspartate aminotransferase, and creatinine levels. The blood samples were analyzed by automatic ADVIA 2400 (Siemens Medical Solutions Diagnostics, Tarrytown, NY) to determine the biochemical plasmatic measures of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), and creatinine in the sham and I3R2 groups at the end of the experiment.

Statistical analysis. Results were compared among the four groups using one-way analysis of variance with a least significant differences post-test. GraphPad Prism software (GraphPad Software Inc, San Diego, Calif) was used for analysis. Results are shown as mean ± standard error of the mean. Results with a value of $P < .05$ were considered statistically significant.

RESULTS

Lactate production in the four experimental groups. As expected, we observed a similar and significant increase of lactate at the end of the 3 hours of ischemia in the groups I3, I3R10′, and I3R2, compared with the sham group ($P < .0001$). Thus, the lactate level was 2.14 ± 0.85 mM in the sham group and 14.19 ± 0.78, 13.77 ± 1.27, and 17.90 ± 1.29 mM, respectively, in the I3, I3R10′, and I3R2 groups (Supplementary Fig 2, online only).

Production of ROS in skeletal muscle during IR. IR is known to produce ROS in skeletal muscle, but ROS kinetics remain to be determined. We found a significant increase in DHE staining in the group sustaining ischemia alone, without reperfusion (I3), compared with sham. Thus, ROS production in skeletal muscle increased by 324.2% ± 66.24%, as inferred from the fluorescence emitted in the I3 group ($P = .038$).

Interestingly, DHE staining decreased when ischemia was followed by 10 minutes of reperfusion. Thus, the fluorescence emitted in the I3R10′ group (146.9% ± 29.76%) was not different from sham values ($P = .92$).

Finally, when ischemia was followed by 2 hours of reperfusion, ROS production in the I3R2 group increased by 349.2% ± 66.66% ($P = .024$) compared with the sham group.

Taken together, these data demonstrate that lower limb IR secondary to aortic clamping is characterized by a biphasic pattern, with both an early and a late increase in ROS production (Fig 1).

Activities of muscle mitochondrial respiratory chain complexes and coupling decreased only after 3 hours of ischemia, followed by 2 hours of reperfusion. We observed an alteration in maximal oxidative capacity (Vmax) only in the group I3R2. The sham, I3, and I3R10′ groups had a similar Vmax of 6.10 ± 0.51, 6.24 ± 0.44, and 5.82 ± 0.49 μmol/min/g. Therefore, no difference was induced by ischemia alone or by ischemia followed by a short-term reperfusion period. However, 2 hours of reperfusion after ischemia decreased significantly the Vmax, relying on complexes I, III, and IV of the mitochondrial respiratory chain (4.24 ± 0.36 μmol/min/g in I3R2; $P = .03$ vs SHAM and $P = .018$ vs I3).

The study of oxygen consumption after stimulation of the complex II by the injection of succinate (Vsucc) did not show any differences among the four groups ($P = .27$). After injection of succinate, the sham, I3, I3R10′, and I3R2 groups had a mitochondrial oxygen consumption of, respectively, 5.12 ± 0.52, 4.76 ± 0.29, 4.14 ± 0.37, and 4.31 ± 0.28 μmol/min/g. Thus, consistent with previous data,[10] complexes II, III, and IV of the mitochondrial respiratory chain appeared more resistant than complex I when exposed to IR.

We found similar results for complex IV (10.64 ± 1.021 μmol/min/g in sham, 12.12 ± 0.81 μmol/min/g in I3, 12.78 ± 1.16 μmol/min/g in I3R10′, and 8.81 ± 0.47 μmol/min/g in I3R2). The decrease in Vtmpd in group I3R2 compared with group I3R10′ ($P = .018$) was likely due to the nonsignificant increase in mitochondrial respiration after 10 minutes of reperfusion compared with sham values.

Finally, we analyzed the ACR, defined by the ratio of ADP-induced oxygen consumption to the basal rate in the absence of ADP. We observed a significant alteration

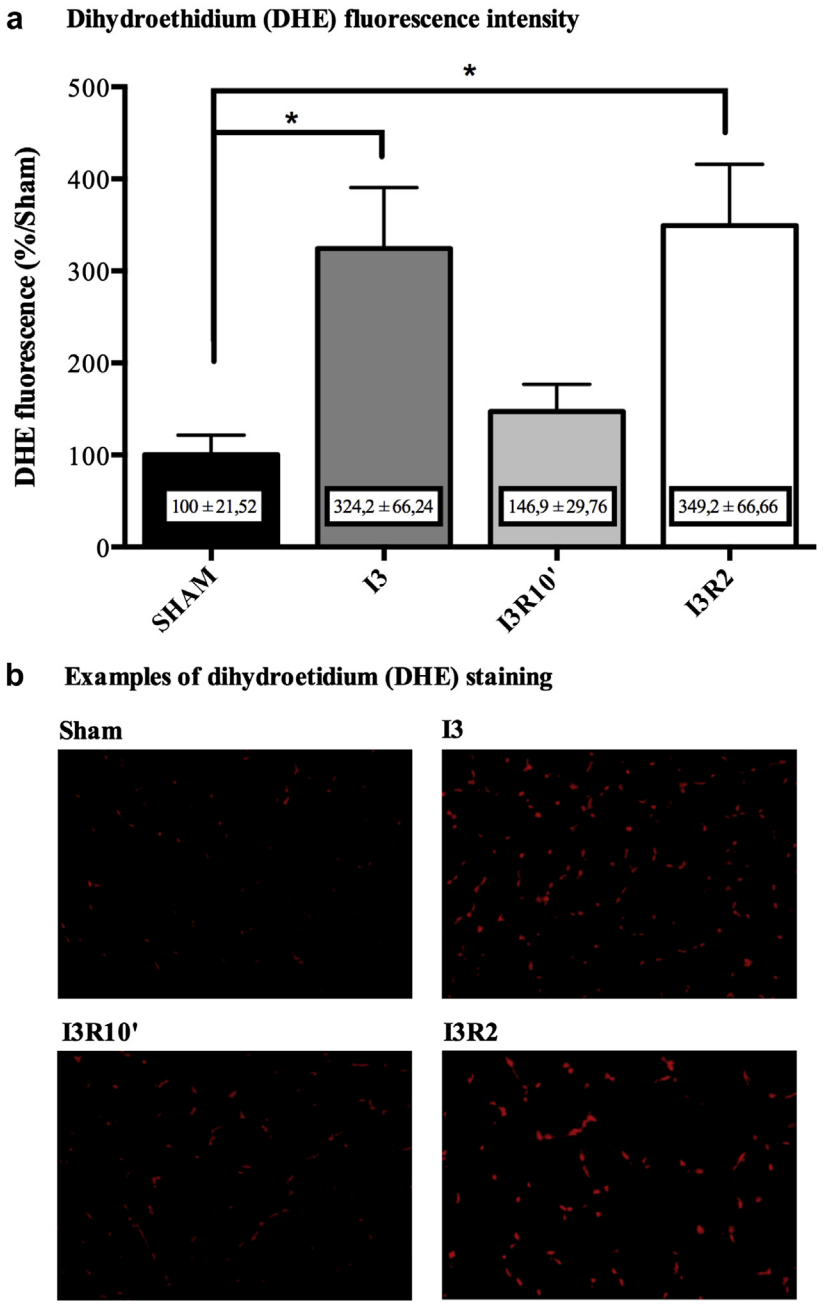

Fig 1. Ischemia alone and ischemia followed by 2 hours of reperfusion enhance reactive oxygen species (ROS) production in skeletal muscles. **a,** Dihydroethidium (*DHE*) fluorescence intensity (expressed as percentage of sham group). The *error bars* indicate the standard error of the mean. **P* < .05. **b,** Examples of DHE staining. *I3,* 3 hours of ischemia; *I3R10′*, 3 hours of ischemia, followed by 10 minutes of reperfusion; *I3R2,* 3 hours of ischemia, followed 2 hours of reperfusion.

of the ACR in group I3R2 compared with the sham group ($P = .009$), group I3 ($P = .038$), and group I3R10′ ($P = .0017$). ACR was 1.937 ± 0.1776 in the sham group, 1.83 ± 0.121 in group I3, 2.072 ± 0.11 in group I3R10′, and 1.33 ± 0.073 in group I3R2.

Taken together, these results support that the mitochondrial respiratory chain at the level of complex I and that mitochondrial coupling is impaired, but only after 3 hours of ischemia, followed by 2 hours of reperfusion (Fig 2).

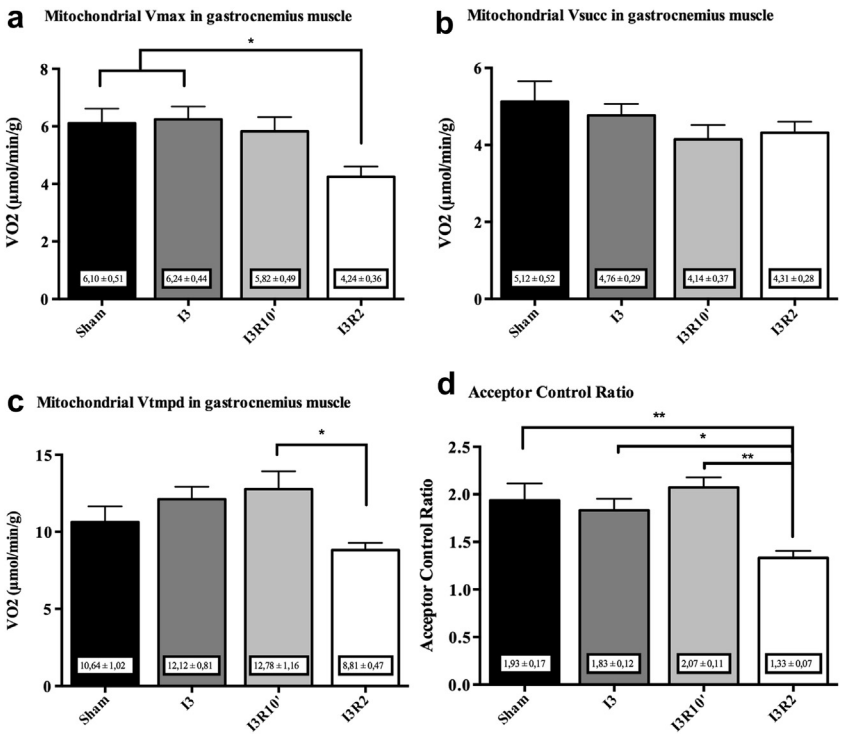

Fig 2. Impaired mitochondrial maximal oxidative capacity and mitochondrial coupling after 3 hours of ischemia and 2 hours of reperfusion. **a,** Maximal fiber mitochondrial respiration rates (*Vmax*) in complexes I, III, and IV. **b,** Combined activity of mitochondrial complexes II, III, and IV after stimulation with succinate (*Vsucc*). **c,** Complex IV activity after stimulation with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (*Vtmpd*). **d,** Acceptor control ratio (ACR). The *error bars* indicate the standard error of the mean. *I3,* 3 hours of ischemia; *I3R10′*, 3 hours of ischemia, followed by 10 minutes of reperfusion; *I3R2,* 3 hours of ischemia, followed 2 hours of reperfusion.*$P < .05$; **$P < .01$.

Remote organs mitochondrial function was not impaired. Results are shown in Fig 3. We did not find any alteration of the mitochondrial respiratory chain complex activities in the different remote organs analyzed. Maximal oxygen mitochondrial consumptions (Vmax) were the same in the sham and I3R2 groups in lung (18.85 ± 2.46 vs 19.89 ± 1.03 µmol/min/g; $P = .69$), heart (125.0 ± 13.86 vs 110.6 ± 7.02 µmol/min/g; $P = .35$), brain (35.83 ± 5.18 vs 34.77 ± 2.76 µmol/min/g; $P = .86$), liver (56.90 ± 6.32 vs 61.39 ± 3.33 µmol/min/g; $P = .56$), and kidney (93.98 ± 8.45 vs 112.5 ± 8.9 µmol/min/g; $P = .15$).

Similarly, stimulation of complex II with succinate (Vsucc) did not show any difference between the sham and I3R2 groups in lung (20.44 ± 2.21 vs 21.34 ± 1.3 µmol/min/g; $P = .79$), heart (95.45 ± 7.89 vs 88.23 ± 4.59 µmol/min/g; $P = .59$), brain (33.22 ± 3.68 vs 32.96 ± 1.7 µmol/min/g; $P = .72$), liver (55.2 ± 21.13 vs 61.89 ± 12.89 µmol/min/g; $P = .45$), and kidney (123.5 ± 8.57 vs 139.3 ± 7.87 µmol/min/g; $P = .19$).

Oxygen mitochondrial consumption after stimulation of complex IV (Vtmpd) was also the same in the sham and I3R2 groups in lung (45.52 ± 3.36 vs 49.84 ± 2.97 µmol/min/g; $P = .51$), heart (241.0 ± 20.01 vs 220.5 ± 12.57 µmol/min/g; $P = .59$), brain (82.60 ± 6.42 vs 79.59 ± 5.5 µmol/min/g; $P = .72$), liver (79.80 ± 8.3 vs 91.81 ± 5.12 µmol/min/g; $P = .23$), and kidney (207.9 ± 13.21 vs 227.0 ± 13.89 µmol/min/g; $P = .69$).

ROS production in remote organs was not increased. Compared with the sham group, there was no significant increase in ROS production in heart (86 ± 27%; $P = .74$), kidney (105 ± 79%; $P = .96$), or liver (34 ± 29%; $P = .29$) after 3 hours of ischemia and 2 hours of reperfusion (Supplementary Fig 3, online only).

Remote organ inflammation was increased in the liver. We did not find tissue histologic alteration, such as necrosis in heart, kidney, and liver, after IR, but liver demonstrated monocyte and macrophages infiltration after 3 hours of ischemia and 2 hours of reperfusion (Supplementary Fig 4, online only).

ALAT, ASAT, and creatinine increased after IR. Compared with sham, IR induced a significant increase of ASAT (97 ± 7 U/L vs 1293 ± 130 U/L; $P < .0001$), ALAT (52 ± 5 U/L vs 330 ± 55 U/L; $P = .0019$), and creatinine (36 ± 4 µmol/L vs 106 ± 10 µmol/L; $P = .0004$; Supplementary Fig 5, online only).

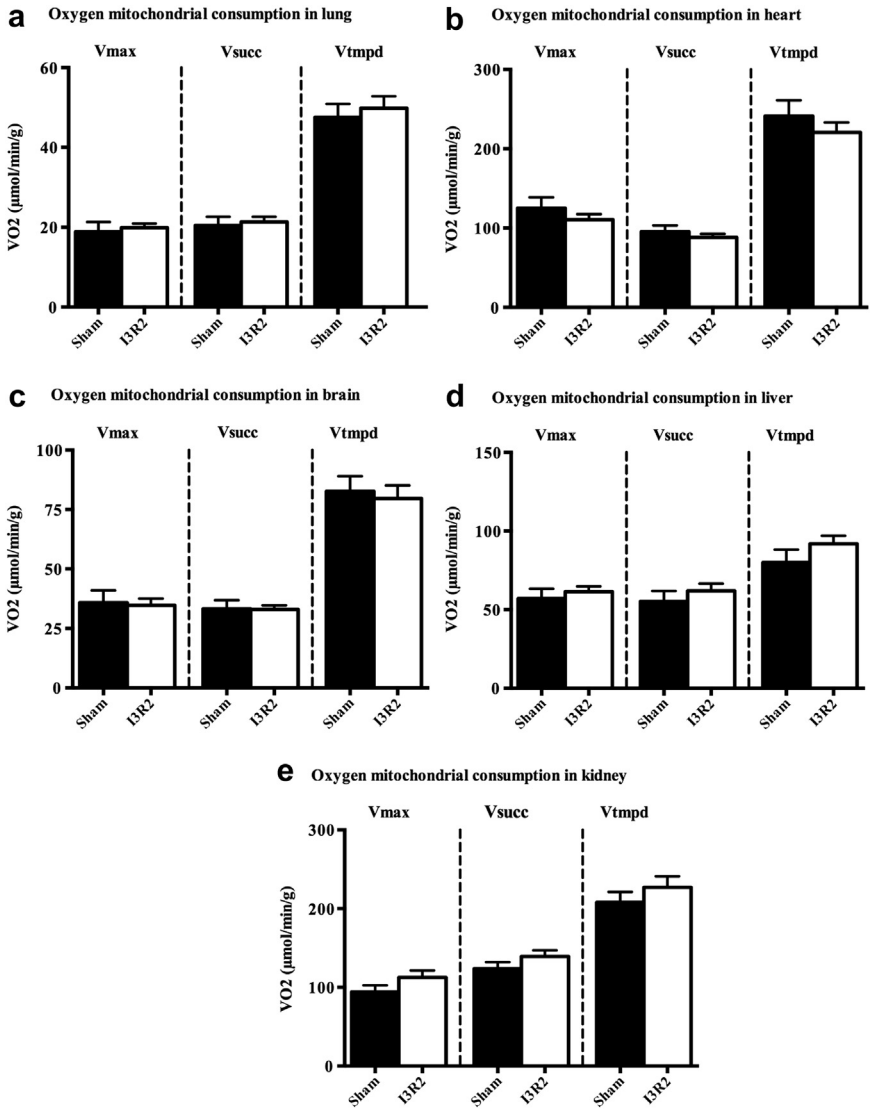

Fig 3. Lack of deleterious effects of lower limb ischemia-reperfusion (IR) on lung, heart, brain, liver, and kidney mitochondrial respiratory chain complexes I, II, III, and IV activities. Volume of mitochondrial oxygen consumption (Vo_2) in **(a)** lung, **(b)** heart, **(c)** brain, **(d)** liver, and **(e)** kidney. *I3,* 3 hours of ischemia; *I3R10′*, 3 hours of ischemia, followed by 10 minutes of reperfusion; *I3R2,* 3 hours of ischemia, followed 2 hours of reperfusion; *Vmax,* maximal fiber mitochondrial respiration; *Vsucc,* activity after stimulation with succinate; *Vtmpd,* activity after stimulation with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride. The *error bars* indicate the standard error of the mean.

DISCUSSION

The main results of this study demonstrate that:

1. There is a biphasic production of ROS, with an early increase during ischemia alone, followed by a normalization after 10 minutes reperfusion and a secondary increase after 2 hours of reperfusion;
2. Skeletal muscle mitochondrial respiratory chain impairment occurs only after 2 hours of reperfusion, and
3. Lower limb IR leading to skeletal muscle impairment is not associated with early mitochondrial dysfunction in the main remote organs such as the heart, lung, liver, kidney, or brain.

Although ROS production is thought to be a key event during lower limb IR-induced deleterious effects, its kinetic remains to be determined, and particularly, ROS production during ischemia remains a debated question. Indeed, lack of oxygen availability during ischemia should preclude ROS formation. Thus, studies have suggested that oxidant production does not occur during ischemia, and for instance, no free radicals were detected during 90 minutes of anoxia in human umbilical vein endothelial cells.

However, other studies have supported the notion that some ROS generation occurs during ischemia.[18-20]

Recently, Vanden Hoek et al[20] demonstrated that cultured cardiomyocytes generate ROS during ischemia. In fact, even after careful artery ligation, hypoxia might not be totally complete because of residual O_2 levels in the tissue.[20,21] Accordingly, ROS production has also been observed during ischemia in the arteriolar wall.[22]

Consistently with these data, we observed an increased DHE staining at the end of the 3 hours of ischemia, before any reperfusion. The mechanisms involved in ROS production are not totally elucidated but should not involve mainly mitochondria that were not impaired by ischemia alone. However, even normal mitochondria produce ROS, and we cannot exclude that a reduced antioxidant defense secondary to ischemia explains such an early increase in ROS production.

The significance of ROS generation during ischemia is an important issue, and data clearly suggest that oxidants generated during ischemia contribute to cell injury during reperfusion[21,23] and progressively erode cellular antioxidant capacity. Although not necessarily creating immediately a lethal stress, antioxidant mechanism depletion participates in cell injuries occurring at reperfusion. Indeed, enhanced ROS production at reperfusion might overwhelm the remaining antioxidant mechanisms.[20,21,24] Such a mechanism might explain the beneficial effects of ROS buffering by ischemic preconditioning, which was observed in experimental and also in clinical lower limb ischemia.[4,7,13]

Interestingly and unexpectedly, we observed ROS normalization after 10 minutes of reperfusion, although significant ROS production during the first minutes of reperfusion has generally been reported.[20,25] Duration of reperfusion might be involved because ROS burst production likely occurs earlier, explaining that the efficiency of ischemic postconditioning deeply relies on its precocity.[25] ROS normalization may also suggest that muscle antioxidant capacities were active and efficacious during the first 10 minutes of reperfusion.

Finally, and importantly, after 2 hours of reperfusion following the 3 hours of ischemia, skeletal muscle DHE staining was increased again, suggesting that ROS buffering was no longer possible. This confirms that enhanced oxidative stress is likely involved in muscle impairment after lower limb IR. At this time, complex I of the mitochondrial respiratory chain might have participated in enhanced ROS production because it was largely impaired and known to participate in ROS release in the setting of IR.[7,9,26] In addition, inflammation favors ROS production, and increased macrophage and monocyte infiltration in skeletal muscle—observed in a similar setting—might have played a role.[10] Furthermore, decreased neutrophil extracellular traps were associated with reduced hind limb IR injury.[27]

Taken together, these data support that ROS production preceded skeletal muscle mitochondrial dysfunction. Indeed, mitochondrial alterations occurred only 2 hours after reperfusion. Acknowledging that inhibitors of ROS production should be used to demonstrate the cause-and-effect of ROS production and mitochondrial dysfunction, a large body of evidence supports that ROS are largely involved in mitochondrial dysfunction during lower limb ischemia.[6,9]

We can therefore conclude that dysfunction of the skeletal muscle mitochondrial respiratory chain is delayed and likely secondary to a previous ROS production. Such data confirm earlier reports from others and our group and support that the mitochondria have a central role in IR.[5-7]

Further, because oxidative stress precedes skeletal muscle mitochondrial dysfunction and arises early during lower limb ischemia, therapy should be conducted, even during ongoing ischemia. Thus, preconditioning being not always feasible, particularly in the context of an emergency, conditioning performed during ischemia (preconditioning) might be a successful approach and deserves to be studied.[28]

Remote organ alterations participate importantly in the prognosis of patients with peripheral artery disease who need surgery. Adembri et al[2] reported that even subtle skeletal muscle dysfunction was associated with lung impairment, leading to multiple organ failure. This was also observed when considering renal function, and recently, Ali et al[4] reported in humans that ischemic preconditioning reduced renal and cardiac alterations associated with aortic clamping.[2,3] This demonstrates that remote organ alteration can be alleviated. We therefore determined potential remote organ impairment and mechanisms by analyzing histology, inflammation markers, biologic, and mitochondrial function.

No significant histologic evidence of injury to the heart, kidney, or liver was observed. This is consistent with previous data supporting that a longer time of reperfusion is needed to induce structural damage.[1,29] Accordingly, DHE staining was not increased in these organs after 2 hours of reperfusion. However, further supporting involvement of inflammation, enhanced macrophage and monocyte infiltration was observed in the liver. Whether hepatic dysfunction might totally explain the ALAT and ASAT increase is unlikely. Indeed, ALAT, ASAT, and increased creatininemia might correspond to skeletal muscle alterations rather than to liver or kidney injuries.[30,31]

To get further insights in this issue, we investigated activities of the mitochondrial respiratory chain complexes in the brain, heart, lung, kidney and liver after lower limb IR, assuming that functional alterations should precede structural alterations. No significant impairment was observed, whatever the mitochondrial respiratory chain complex or organ studied.

Such a precise analysis at the mitochondrial level has never been reported before and suggests that remote organ alteration did not begin that early at such a subcellular level. Our results may be explained by the relatively short reperfusion period, mitochondrial alterations in remote organs relying partly on inflammation that possibly needs

more time to be initiated, or more precise measurement methods, or both.[27,32,33]

CONCLUSIONS

The activities of the mitochondrial respiratory chain complexes of brain, lung, kidney, heart, and liver were not impaired after 3 hours of aortic clamping and 2 hours of reperfusion. However, skeletal muscle IR was associated with an early local production of ROS and later with mitochondrial dysfunction. Such a kinetic, described for the first time, supports that reducing significantly ROS release, even during ischemia, should result in better muscle protection. Thus, therapy should be conducted early in the setting of lower limb ischemia, and because preconditioning is not always feasible, studies are needed to determine whether ischemic preconditioning might be a successful approach.

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AUTHOR CONTRIBUTIONS

Conception and design: MG, AC, TC, JZ, BG
Analysis and interpretation: MG, AC, TC, JB, AM, FS, BG
Data collection: MG, AC, TC, JB
Writing the article: MG, AC, BG
Critical revision of the article: TC, JB, AM, JZ, FS
Final approval of the article: MG, AC, TC, JB, AM, JZ, FS, BG
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Overall responsibility: BG

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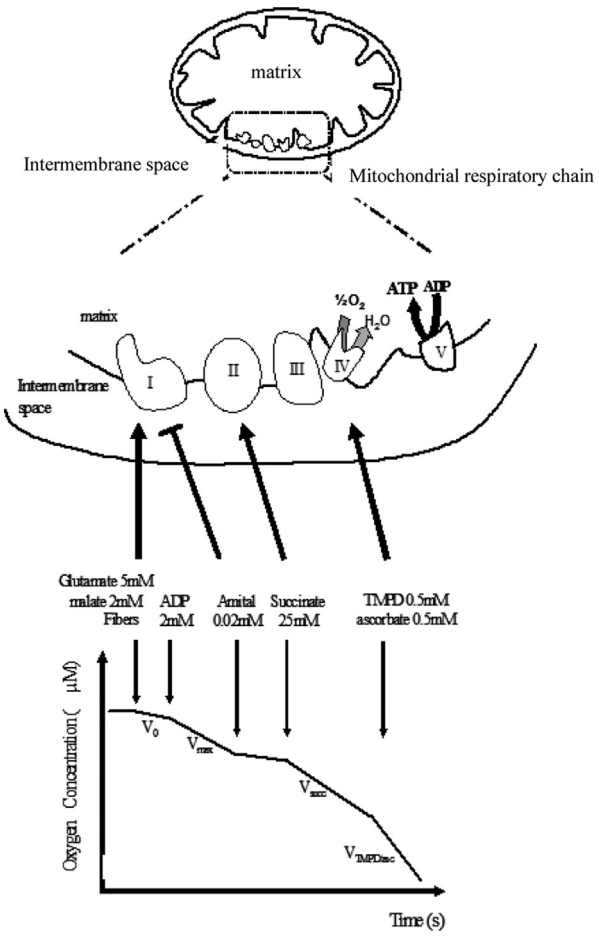
SUPPLEMENTARY METHODS (online only).

Detection of reactive oxygen species. To detect the presence of reactive oxygen species (ROS) in skeletal muscle, heart, kidney, and liver, serial 10-μm sections were cut on a cryostat microtome, mounted onto glass slides, and incubated with 2.5 μmol/L of dihydroethidium (DHE).[14] DHE was used in heart, kidney, and liver only in the sham and the I3R2 (ischemia for 3 hours, followed by reperfusion for 2 hours) groups at the end of the experiment. In skeletal muscle, DHE was used in the four groups at the end of the experiment. DHE produces red fluorescence when oxidized to ethidium bromide by ROS, including superoxide anion.[15] After staining, sections were examined under an Eclipse E800 epifluorescence microscope (Nikon, New York, NY), and the emission signal was recorded with a Zeiss 573-637-nm filter (Carl Zeiss, Oberkochen, Germany). Pictures taken with the microscope were analyzed with Photoshop CS2 image processing software (Adobe Systems, San Jose, Calif).

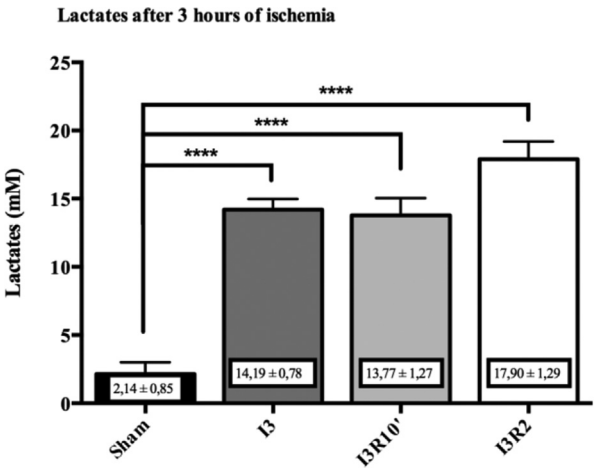
Immunohistochemical procedure. To detect inflammation in heart, kidney, and liver in the sham and I3R2 groups at the end of the experiment, we used monocyte macrophage-2 (Millipore, Billerica, Mass) antibody on 10-mm-thick serial sections of tisuse.[10] Briefly, muscle sections were air dried and then fixed in paraformaldehyde 4% for 3 minutes. The sections were placed in a 2% solution of hydrogen peroxide for 5 minutes, which served to reduce endogenous or pseudoperoxidase background staining. Primary antibody was used at predetermined optimal dilutions. A standard indirect immunoperoxidase procedure, using biotinylated goat antirat antibody (Millipore) and streptavidin-horseradish peroxidase (Millipore), was used to detect binding of monocyte macrophage-2. The slides were developed by diaminobenzidine substrate. Tissues were counterstained briefly with hematoxylin and eosin before mounting.

Permeabilization of muscle and heart fibers. Mitochondrial respiration was studied in saponin-skinned fibers, as previously described.[16,17] Briefly, fibers were separated, permeabilized, in a bath of solution containing 50 mg/mL saponin for 30 minutes at 4°C, under shaking. The permeabilized fibers were then washed for 10 minutes under shaking to remove saponin. Fibers were placed in a bath with the respiratory solution for 5 minutes twice to remove any phosphates.

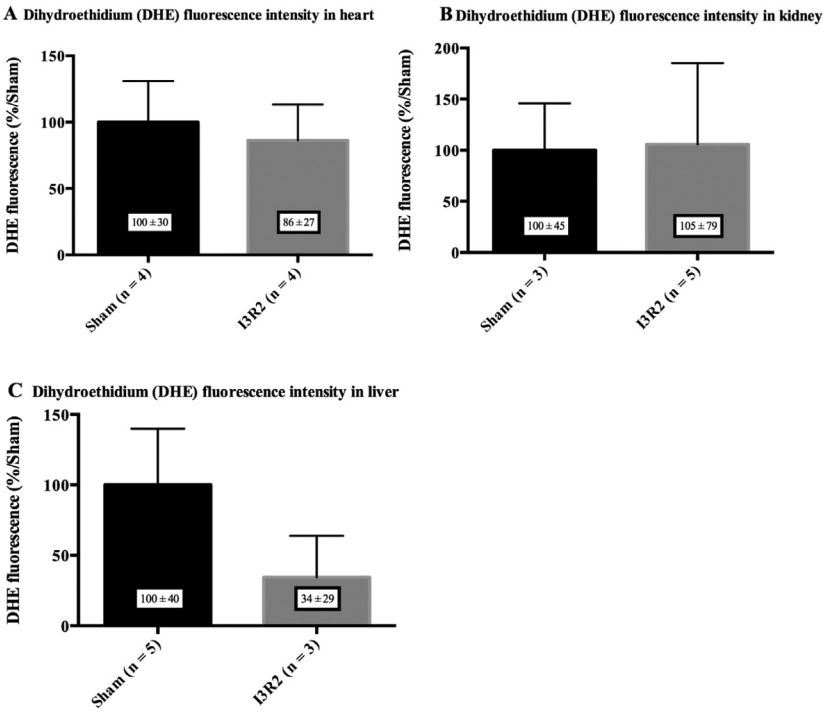
Isolation of mitochondria in lung, heart, brain, liver, and kidney. Lung, heart, brain, liver, and kidney mitochondria were isolated on ice from animals. Tissue was placed in isolation buffer containing 70 mM sucrose, 210 mM mannitol, and 1 mM ethylene glycol tetraacetic acid in 50 mM Tris/HCl (pH 7.4). The tissue was finely minced with scissors and then homogenized in the same buffer using a tissue grinder. The homogenate was centrifuged at 3000 rpm for 3 minutes at 4°C and the resultant supernatant at 10,000 rpm for 10 minutes at 4°C. The mitochondrial pellet was washed in isolation buffer containing 70 mM sucrose and 210 mM mannitol in 50 mM Tris/HCl (pH 7.4) before being resuspended in this buffer. Aliquots were then removed for protein measurement by the Bradford method (Biomate 3; Thermo Fisher Scientific Inc, Waltham, Mass).



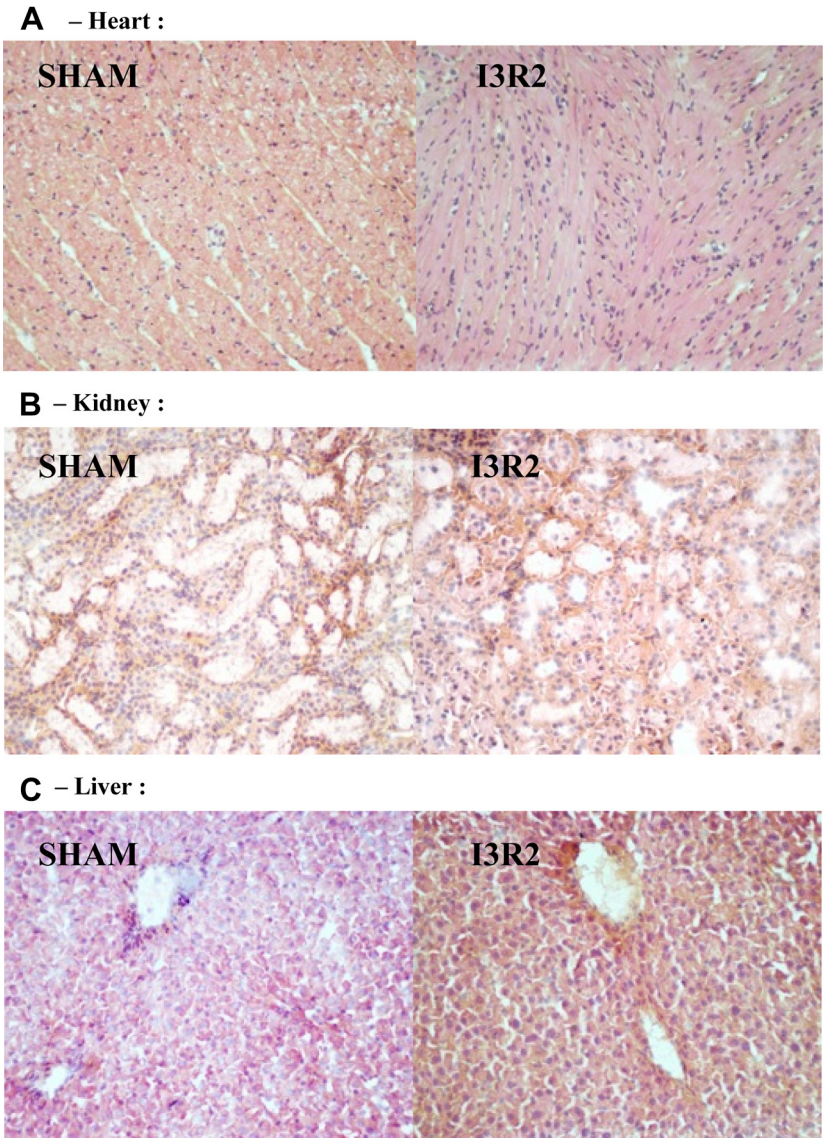
Supplementary Fig 1 (online only). Top, Schematic representation shows the mitochondrial respiratory chain with specific substrates and inhibitors. *ADP,* adenosine diphosphate; *I,* complex I (nicotinamide adenine dinucleotide reductase [NADH]-coenzyme Q [CoQ] reductase); *II,* complex II (succinate-CoQ reductase); *III,* complex III (CoQH2-c reductase); *IV,* complex IV (cytochrome-c oxidase, COX); *V,* adenosine triphosphate (*ATP*) synthase; *TMPD, N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride. **Bottom,** Schematic oxygraph trace shows oxygen consumption by the permeabilized skeletal myofibers, using indicated substrates and inhibitors: V_o, before ADP; V_{max}, complexes I, III, IV activities, using glutamate and malate; V_{succ}, complexes II, III, IV activities, using succinate; V_{TMPD}, complex IV activity using TMPD/ascorbate.



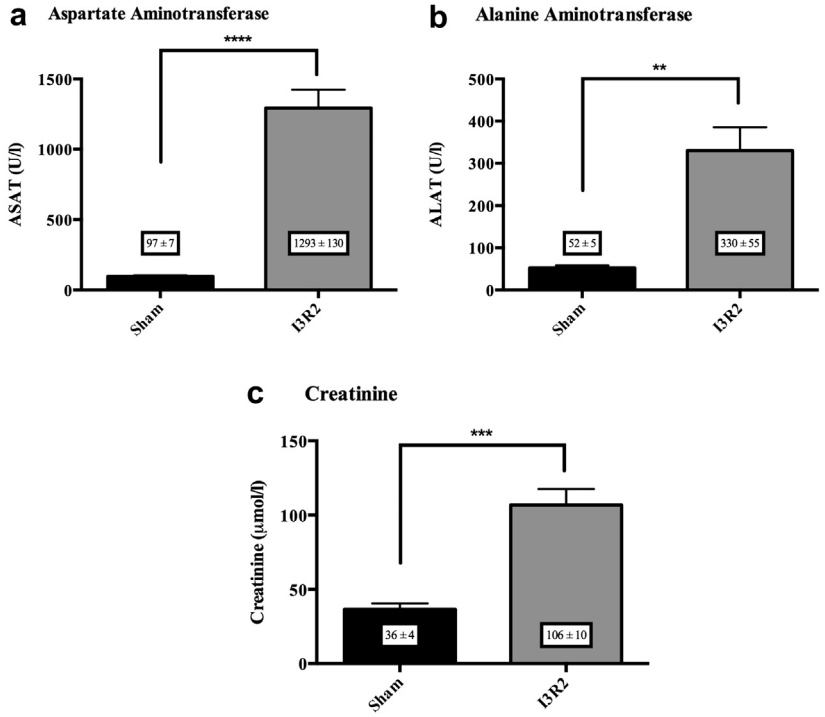
Supplementary Fig 2 (online only). Lactate production after an ischemia of 3 hours. The *error bars* indicate the standard error of the mean. *I3,* 3 hours of ischemia; *I3R10′,* 3 hours of ischemia, followed by 10 minutes of reperfusion; *I3R2,* 3 hours of ischemia, followed 2 hours of reperfusion.**** $P < .0001$.



Supplementary Fig 3 (online only). Dihydroethidium (*DHE*) fluorescence intensity (expressed as percentage of sham). Ischemia for 3 hours, followed by 2 hours of reperfusion (*I32H*) does not enhance reactive oxygen species (ROS) production in **(A)** heart, **(B)** kidney and **(C)** liver.



Supplementary Fig 4 (online only). Compared with sham, 3 hours of ischemia and 2 hours of reperfusion (*I3R2*) of skeletal muscle activates inflammation in **(C)** liver but not **(A)** in heart or **(B)** kidney (original magnification ×200).



Supplementary Fig 5 (online only). Three hours of ischemia, followed by 2 hours of reperfusion (*I3R2*), increases circulating **(a)** aspartate aminotransferase (*ASAT*), **(b)** alanine aminotransferase (*ALAT*), and **(c)** serum creatinine. The *error bars* indicate the standard error of the mean. $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.