Metformin prevents ischemia reperfusion-induced oxidative stress in the fatty liver by attenuation of reactive oxygen species formation

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1Center for Experimental Medicine, Department of Metabolism and Diabetes, Charles University, Prague, Czech Republic; 2Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic; 3Clinical and Transplant Pathology Department, Charles University, Prague, Czech Republic; 4Department of Diagnostic and Interventional Radiology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic; 5Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic; 6Department of Physiology, Charles University in Prague, Faculty of Medicine in Hradec Kralove, Hradec Kralove, Czech Republic; 7Department of Chemistry, University of Cambridge, Cambridge, United Kingdom; and 8Institute of Inorganic Chemistry Academy of Science CR, Husinec-Rez, Czech Republic

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Metformin prevents ischemia reperfusion-induced oxidative stress in the fatty liver by attenuation of reactive oxygen species formation. Am J Physiol Gastrointest Liver Physiol 309: G100–G111, 2015. First published June 4, 2015; doi:10.1152/ajpgi.00329.2014.—Nonalcoholic fatty liver disease is associated with chronic oxidative stress. In our study, we explored the antioxidant effect of anti diabetic metformin on chronic [high-fat diet (HFD)-induced] and acute oxidative stress induced by short-term warm partial ischemia-reperfusion (I/R) or on a combination of both in the liver. Wistar rats were fed a standard diet (SD) or HFD for 10 wk, half of them being administered metformin (150 mg·kg body wt−1·day−1). Metformin treatment prevented acute stress-induced necroinflammatory reaction, reduced alamine aminotransferase and aspartate aminotransferase serum activity, and diminished lipoperoxidation. The effect was more pronounced in the HFD than in the SD group. The metformin-treated groups exhibited less severe mitochondrial damage (markers: mitochondrial depolarization, cytochrome c release, citrate synthase activity, mtDNA copy number, mitochondrial respiration) and apoptosis (caspase 9 and caspase 3 activation). Metformin-treated HFD-fed rats subjected to I/R exhibited increased antioxidant enzyme activity as well as attenuated mitochondrial respiratory capacity and ATP resynthesis. The exposure to I/R significantly increased NADH- and succinate-related reactive oxygen species (ROS) mitochondrial production in vitro. The effect of I/R was significantly alleviated by previous metformin treatment. Metformin downregulated the I/R-induced expression of proinflammatory (TNF-α, TLR4, IL-1β, Ccr2) and infiltrating macrophage (Ly6c) and macrophage (CD11b) markers. Our data indicate that metformin reduces mitochondrial performance but concomitantly protects the liver from I/R-induced injury. We propose that the beneficial effect of metformin action is based on a combination of three contributory mechanisms: increased antioxidant enzyme activity, lower mitochondrial ROS production, and reduction of postischemic inflammation.

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Metformin is an oral antihyperglycemic drug widely used in the treatment of Type 2 diabetes (T2D) (7). There is evidence that metformin also protects against oxidative stress in various tissues, although the antioxidant activity of metformin is not well understood. It has been shown that hyperglycemia is associated with increased reactive oxygen species (ROS) formation due to superoxide overproduction from the mitochondrial electron transport chain as a consequence of increased glycolysis (8). It has been hypothesized that the antioxidative effect of metformin is simply the consequence of its glucose-lowering effect and subsequent decrease in superoxide anion production (3). Nevertheless, metformin has also displayed antioxidative characteristics in several models of oxidative stress without hyperglycemia (2, 19, 20, 21, 62).

Increased production and/or ineffective scavenging of ROS play an important role in the pathogenesis of many diabetic complications (14). Nonalcoholic fatty liver disease, a component of a cluster of pathophysiological conditions preceding the manifestation of overt T2D, is often associated with chronic oxidative stress. Surprisingly, there is not much data regarding the effect of metformin on oxidative stress in the fatty liver in the context of insulin resistance.

Liver ischemia-reperfusion (I/R) injury is a major contributor to tissue damage during liver transplantation, liver resection procedures, hypovolemic shock, and trauma. It is a major cause of primary nonfunction after liver transplantation (11). I/R injury is a phenomenon in which damage to a hypoxic organ is accentuated following the return of oxygen delivery. The immediate response involves the disruption of the cellular mitochondrial oxidative phosphorylation and accumulation of metabolic intermediates during the ischemic period and oxidative stress following the resumption of blood flow (42). As a result, direct cellular damage occurs due to the ROS impact on mitochondria. Moreover, oxidative stress impairs mitochondrial function, which leads to further production of ROS and amplification of oxidative stress and tissue injury (37). ROS produced during oxidative stress may also act as signaling molecules, which activate inflammatory pathways and which in turn accentuate primary injury (32). Thus the extent of early ROS formation represents a critical event in terms of the magnitude of the final injury.
The aim of our study was to assess the effect of metformin on both chronic oxidative stress induced by high-fat diet (HFD) feeding and acute stress caused by short-term warm IR, or on the combination of both. We recorded the effects of metformin on whole body, tissue, and cellular levels. We further focused on the possible mechanisms of metformin antioxidant action, particularly on those related to metformin interaction with the mitochondrial respiratory chain, ROS production, and inflammatory processes.

MATERIALS AND METHODS

Animals and experimental design. Male Wistar rats aged 4 mo were kept in a temperature-controlled environment with a 12-h light-dark cycle on either standard diet (SD; protein 33cal%, starch 58cal%, fat 9cal%) or HFD (protein 28cal%, starch 12cal%, fat 60cal%) diets. Half of the animals in both groups were provided metformin in a dose of 150 mg/kg body wt for 10 wk by gavage. This dose corresponds to a human dose of 15 mg·kg⁻¹·day⁻¹, according to the normalization of body surface area (18), and results in comparable serum metformin concentrations (1–10 μM). The animals in groups labeled IR were subjected to short-term (20 min) warm partial ischemia induced during anesthesia by portal vein ligation. After restoration of circulation, the abdominal cavity was closed and animals were left to recover 48 h prior to decapitation. Sham-operated animals were subjected only to the opening of the abdominal cavity without portal vein ligation. All experiments were performed in accordance with the Animal Protection Law of the Czech Republic 311/1997, which is in compliance with the Principles of Laboratory Animal Care (NIH Guide to the Care and Use of Laboratory Animals, 8th edition, 2013) and were approved by the Ethical Committee of the Institute for Clinical and Experimental Medicine.

Histological evaluation of liver injury. Liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin blocks, and routinely processed. Sections cut at 4–6 μm were stained with hematoxylin and eosin and examined with an Olympus BX41 light microscope.

Electrophoretic separation and immunodetection. The homogenate and postmitochondrial fractions were prepared from freshly excised liver tissue. The protein concentration was determined by the BCA Protein Assay Reagent (Thermo Scientific, Waltham, MA). The proteins were separated by electrophoretic separation under denaturing conditions and electroblotted to PVDF membranes. Proteins of interest were detected by using specific antibodies (cytochrome c: ab53056 Abcam, Cambridge, UK; caspase 3: no. 9662, caspase 9: no. 9506, Cell Signaling, MA). The loading control was performed with rabbit polyclonal antibody to β-actin (ab8227, Abcam, Cambridge, UK). Bands were visualized by using ECL and quantified by using the FUJI LAS-3000 imager (FUJI FILM, Japan) and Quantity One software (Bio-Rad, Hercules, CA).

Citrate synthase specific activity. Citrate synthase (CS) activity was measured according to Rustin et al. (52). Briefly, the cells were lysed with 1% lauryl maltoside and CS activity was measured according to the conversion of acetyl-CoA and oxaloacetate to citrate and CoA. This rate-limiting reaction catalyzed by CS was coupled to the irreversible reduction of chromogenic substrate TNB (thionitrobenzolic acid). Citrate synthase activity was measured as an increase of irreversible reduction of chromogenic substrate TNB (thionitrobenzoic acid).

Measurement of mitochondrial respiration by the Seahorse technique. An XF 24 extracellular flux analyzer was used to determine mitochondrial function (XF 24, Seahorse Bioscience, http://www.seahorsebio.com/company/about.php). The validity of this method compared with classical Clark electrode oxygen graph measurements has been proved recently (22). Freshly isolated liver mitochondria (10 μg per well) in 50 μl of MAS buffer (220 mM mannitol, 70 mM sucrose, 10 mM KH2PO4, 5.5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, pH 7.4) were delivered to each well on ice and the 24-well plate was centrifuged at 21,000 g for 10 min. The resultant pellet was washed three times in MSH buffer to get rid of matrix components and stored at −80°C.

Fluorometric determination of reactive oxygen species production. ROS production in SMPs in vitro was measured by using a DCFDA (Molecular Probes, Eugene, OR) dye. Briefly, the assay was performed with ~0.2 mg of mitochondrial protein per milliliter in an MAS buffer supplemented with either 10 mM NADH, 10 mM glycerol phosphate (sn-glycerol 3-phosphate), or 10 mM succinate. The final concentration of DCFDA was 10 μM. Subsequently, the following substances were added in the indicated order (final concentrations): 10 mM glutamate + 5 mM malate; 4 mM ADP, 2 μM rotenone; 10 mM succinate. The data are given as the oxygen consumption rate (OCR) in picomoles O2 per minute.

Parameters of oxidative stress. The activities of superoxide dismutase (SOD), catalase, glutathione peroxidase 1 (GPx1) and thiobarbituric acid-reactive substances (TBARS) content were determined as described previously (50). The content of reduced GSH was determined by using the Glutathione HPLC diagnostic kit (Chromsystems, Munich, Germany). 4-hydroxynonenal (4-HNE) concentration by OxiSelect HNE Adduct Competitive ELISA Kit (Cell Biolabs, San Diego, CA).

The relative amount of mitochondrial to nuclear DNA was determined by normalized Nd5 to Ucp2 levels.

Isolation of liver mitochondria and preparation of submitochondrial particles. Liver mitochondria were prepared by differential centrifugation as described by Bustamante et al. (10) with some modifications. Rat liver tissue was homogenized at 0°C by using a Teflon-glass homogenizer as a 10% homogenate in a medium containing 220 mM mannitol, 70 mM sucrose, and 1 mM HEPES, pH 7.2 (MSH medium). Crude impurities were removed by centrifugation at 800 g, 10 min, and the remaining supernatant was centrifuged for 10 min at 8,000 g. Pelleted mitochondria were resuspended in the MSH medium, washed twice via 10 min centrifugation at 8,000 g, and finally resuspended in a concentration of 20–30 mg protein/ml. Mitochondrial proteins were determined by the BCA method (Thermo Scientific, Waltham, MA). To determine ROS production, submitochondrial particles (SMP) were prepared according to Ide et al. (27).

EPR spin-trapping measurements. Measurement of free radicals by electron paramagnetic resonance (EPR) is based on the ability of radical compounds to absorb microwave energy in strong magnetic fields. To demonstrate the effect of IR and metformin on mitochondrial ROS production, an aliquot of the SMP suspension (2 mg protein) was reacted with 10 mM NADH and the spin-trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) in a Quartz flat cell with a thickness of 0.5 mm. Immediately after the start of the reaction, EPR spectra were recorded at an ambient temperature (25°C) using an EPR spectrophotometer (Bruker, Karlsruhe, Germany), scanning 300–10,000 G with an 80 μs sweep time and an 8192 point resolution.
E-540 spectrometer operating at X-band (9.7 GHz). Microwave power was 20 mW and magnetic field modulations were 1 and 4 Gauss. The quantification of the signals’ intensities was performed by comparing the amplitude of the observed signal with the standard Mn$^{2+}$/Zn$^{2+}$ and Cr$^{3+}$/MgO markers. EPR spectra were recorded as the first derivatization and the main parameters ($g$-factor values, hyperfine coupling constant A, line widths $\Delta$Hpp (peak-to-peak distance) and $\Delta$App (peak-to-peak amplitude)) were calculated according to Weil and Bolton (68). The DMPO adducts were identified according to amplitude (51).

Weil and Bolton (68). The $Cr_{3}^{2+}$ access the portal vein, and a remote block of tuning capacitors set tuned$^{31}P/1H$ coil. The coil consisted of two parts: a sole probe head cm apart from the probe head to reduce the influence of the weight and tuning torque reaction forces of the bulky tuning capacitors on the observed liver. During the nuclear magnetic resonance (NMR) experiment, the animals lay on the back in a holder rig with the coil on the top of the exposed liver. After acquisition of the first spectrum, the portal vein was occluded and the subject was repositioned to the same position in the magnet. Spectra were then acquired for the next 20 min. The occlusion was then released and 18 more spectra were acquired in 5-min intervals. A single-pulse sequence without proton decoupling was used to acquire the $^{31}P$ spectra with the following parameters: TR = 500 ms, 512 averages. Relative ATP levels were then evaluated from intensities of $\beta$-ATP peaks using the jMRUI software package (63).

Real-time quantitative PCR analysis. Total RNA was extracted from rat liver tissue by using an RNeasy Mini Kit (Qiagen, Courtaboeuf, France); 500 ng of total RNA was reverse-transcribed by use of a High Capacity Reverse Transcription Kit (Life Technologies, Forest City, CA) and real-time quantitative PCR analysis (RT-qPCR) was performed (viia7, Life Technologies, Forest City, CA) with EvaGreen qPCR Mix (Solis BioDyne). The primers were designed with Primer3 software; details are given in Table 1. Gene expression values were normalized to the expression value of the housekeeping genes $Gush$ and $B2m$. Calculations were based on the comparative cycle threshold Ct method ($2^{-\Delta\Delta Ct}$).

Biochemical analyses. Nonesterified fatty acids (FFA), triacylglycerol, glucose, lactate, and $\beta$-hydroxybutyrate serum content were determined with commercially available kits (FFA: FFA half micro test, Roche Diagnostics, Mannheim, Germany; triglycerides, cholesterol, and glucose: ERBA-Lachema, Brno, Czech Republic; $\beta$-hydroxybutyrate: Cayman Chemical, Ann Arbor, MI; lactate: Biovision, San Francisco, CA). Serum glucose was measured using an AccuChek glucometer (Roche, Czech Republic). Glycogen and triacylglycerol content in the liver were determined as described previously (12).

Statistical analyses. Data are presented as means $\pm$ SE of multiple determinations. Statistical analyses were performed with SigmaStat (Systat Software, San Jose, CA) or Microsoft Excel statistical packages. One-way ANOVA with Bonferroni or two-way ANOVA with multiple determinations was employed to test the differences between more than two groups. The Student’s t-test was used to detect the effect of I/R within groups. Differences were considered statistically significant at the level of $P < 0.05$.

RESULTS

Metabolic characteristics of the experimental groups. Table 2 summarizes the effects of HFD administration and metformin treatment on selected metabolic characteristics. As expected, HFD led to the elevation of serum concentrations of lactate and nonesterified fatty acids and to the increase in ketogenesis. Metformin had no significant effect on most of the parameters despite the enhanced lactate production in both SD and HFD groups. However, metformin administration resulted in diminished weight gain in the HFD group. Liver triacylglycerol content was elevated and glycogen content reduced in the HFD group, but the effect of the diet was not reversed by metformin. To estimate the degree of liver injury induced by oxidative stress, we measured the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities after I/R (Table 3). As expected, I/R led to a significant increase of serum AST and ALT, and this effect was more pronounced in HFD-fed animals. Metformin had no significant effect on most of the parameters despite the enhanced lactate production in both SD and HFD groups. However, metformin administration resulted in diminished weight gain in the HFD group. Liver triacylglycerol content was elevated and glycogen content reduced in the HFD group, but the effect of the diet was not reversed by metformin.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Ref. Seq.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Arg1</td>
<td>M_017134.1</td>
<td>CTGCTGGGAAGAAGAAAGAAG</td>
<td>TTTCCCAAGAGTTGGTTCAC</td>
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<td>Emr1</td>
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<td>TGGCAGGAAATCAAGGATTC</td>
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<tr>
<td>Mrc1</td>
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<td>GAAACAGACTGGAGAAAGG</td>
<td>AGCTGGGCTTTGAGGTATC</td>
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<tr>
<td>TLR4</td>
<td>NM_019718.1</td>
<td>GGATTAGCAGATGAAATAGG</td>
<td>GTCDDCAAGCAAGGAGGAT</td>
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<tr>
<td>Osp</td>
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<td>TGAGTAAGACATTCGAGTAG</td>
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<td>Ccr2</td>
<td>NM_021866.1</td>
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<tr>
<td>IL10</td>
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<td>TLR4</td>
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<td>Ly6-C</td>
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<td>Tgf1b</td>
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<tr>
<td>TNFα</td>
<td>NM_012675.3</td>
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<td>mtNdS</td>
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<tr>
<td>Ucp2</td>
<td>NC_0015900.4</td>
<td>GGGGGAGAGAGATGAGAGAGA</td>
<td>ATGGGAGAGATGAGAGAGA</td>
</tr>
</tbody>
</table>

Ref. Seq., reference sequence.
subjected to I/R injury showed mild predominantly centrilobular steatosis without any significant necroinflammatory reaction. The HFD itself increased lipid peroxidation measured as TBARS or 4-HNE content in the liver (Fig. 1, B and C). For TBARS, I/R comparably increased lipoxygenase formation in both groups. Importantly, metformin administration diminished TBARS formation by 30 and 25% in the SD and HFD groups, respectively. 4-HNE content was increased only in the HFD, but not in the SD group subjected to I/R. Metformin mitigated the combined effect of the HFD and I/R and reduced 4-HNE content ~2.5 times. These results indicate that metformin protects liver tissue against acute oxidative stress in vivo, probably by preventing excessive ROS formation or strengthening the antioxidant defense.

**Metformin reduces oxidative stress-induced mitochondrial damage and apoptosis in vivo.** Lower oxidative stress may result in less severe mitochondrial damage in metformin-treated animals exposed to stress conditions compared with untreated ones. In the liver exposed to I/R, we found increased cytochrome c content in cytosol (SD< HFD), suggesting its leakage from the outer mitochondrial space (Fig. 2A). This was substantially diminished in metformin-treated animals, thus indicating less mitochondrial injury. Cytosolic cytochrome c is known to trigger apoptotic cell death, and we further wanted to confirm whether the reduced/increased mitochondrial damage would be reflected by the changes in the intensity of cell death executed by the apoptotic pathway. Therefore, we determined the activation of caspase 9 and its downstream target caspase 3 in liver homogenates. I/R resulted in caspase 9 and caspase 3 activations in both SD and HFD groups, the effect being more pronounced in the latter. Metformin administration eradicated (SD) or significantly attenuated (HFD) proapoptotic signaling in both groups (Fig. 2, B and C). To assess whether the antiapoptotic effect of metformin is translated into the quantitative preservation of mitochondria, we determined the activity of citrate synthase which an exclusive mitochondrial matrix enzyme. Figure 2D demonstrates that I/R resulted in the reduction of citrate synthase activity in both the SD and HFD groups and that the effect of I/R was prevented by metformin. The presence of mitochondrial damage and the protective effect of metformin were independently confirmed by the direct determination of mtDNA copy number in all experimental groups (Fig. 2E). In the HFD group, the effect of metformin and I/R on mitochondrial function was assessed by measuring mitochondrial respiration of NADH-dependent (Fig. 3A) and FADH-dependent substrates (Fig. 3B). In the basal state, i.e., without addition of any substrates and in the presence of malate and glutamate only (NADH-dependent substrates), we did not find any difference between the groups. In contrast, the maximal respiration rate (OCR), measured after the addition of ADP, was significantly diminished both by I/R and metformin administration, but this effect was not additive. In other words, although mitochondria from metformin-treated animals exhibited lower OCR compared with the untreated ones, they were not further damaged by the exposure to I/R. With succinate used as a substrate (FADH-dependent), the OCR was not affected by metformin treatment or I/R injury. Taken together, these data indicate that metformin treatment protects liver mitochondria from I/R-induced mitochondrial damage, which further ameliorates apoptosis and partly preserves mitochondrial function.

**Effect of metformin on antioxidative defense status in vivo:** The previous data demonstrate that metformin significantly alleviates oxidative stress evoked by the HFD itself or, more pronouncedly, in combination with I/R. Subsequently, we tested whether the effect of metformin is mediated by the increased activity of antioxidative mechanisms. In the liver, HFD administration itself was associated with diminished GSH stores and decreased activities of GPx1 and SOD, whereas catalase activity was upregulated (Table 4). Metformin treatment ameliorated some of these parameters (GSH content and SOD activity) affected by the diet alone. In the HFD group, oxidative stress evoked by I/R resulted in further exhaustion of liver GSH content and in reduction of antioxidative enzyme activity by 25–50% compared with the values in nonischemic animals. Metformin treatment led to partial restoration of GSH content and enzyme activity. We did not observe any effect of metformin on the expression of relevant genes that encode antioxidative enzymes (not shown). The presented data dem-

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**Table 2. Metabolic characteristics of the experimental groups**

<table>
<thead>
<tr>
<th></th>
<th>SD</th>
<th>SD + Met</th>
<th>HFD</th>
<th>HFD + Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>482 ± 9</td>
<td>466 ± 12</td>
<td>490 ± 7</td>
<td>437 ± 10f</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metformin, µmol/l</td>
<td>6.2 ± 0.8</td>
<td>6.1 ± 0.8</td>
<td>6.1 ± 1</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>Glycemia, mmol/l</td>
<td>6.4 ± 0.1</td>
<td>6.2 ± 0.2</td>
<td>6.1 ± 0.1</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>3.5 ± 0.2 b</td>
<td>4.7 ± 0.3 a</td>
<td>5.9 ± 0.5 b</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>Triacylglycerol, mmol/l</td>
<td>1.8 ± 0.09 c</td>
<td>1.8 ± 0.2</td>
<td>1.4 ± 0.12 c</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>0.75 ± 0.05 a</td>
<td>0.81 ± 0.04</td>
<td>1.02 ± 0.05 a</td>
<td>0.91 ± 0.1</td>
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<tr>
<td>Cholesterol, mmol/l</td>
<td>1.6 ± 0.11</td>
<td>1.4 ± 0.13</td>
<td>1.5 ± 0.17</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>β-OH butyrate, mmol/l</td>
<td>0.17 ± 0.2 e</td>
<td>0.23 ± 0.01</td>
<td>0.94 ± 0.2 e</td>
<td>1.03 ± 0.03</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerol, µmol/g</td>
<td>12.0 ± 1.3 e</td>
<td>9.1 ± 1.1 f</td>
<td>60.5 ± 11.3 e</td>
<td>53.9 ± 8.6</td>
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<tr>
<td>Glycogen, µmol glucose/g</td>
<td>280 ± 13 b</td>
<td>277 ± 20</td>
<td>219 ± 28 b</td>
<td>185 ± 14</td>
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</tbody>
</table>

All parameters were determined in the serum or liver of fed animals. Data are expressed as means ± SE. Serum parameters: n = 20 per group (blood was collected prior operation); liver parameters: n = 10 per group (only sham-operated animals). SD, standard diet; Met, metformin; HFD, high-fat diet; FFA, free fatty acids. Values marked with the same letter are statistically significantly different; 0.001; a,b,c,d,e,f,g P < 0.05; h,i,j,k,l,m,n,o P < 0.01.

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**Table 3. Effect of ischemia reperfusion on the markers of liver injury**

<table>
<thead>
<tr>
<th></th>
<th>SD</th>
<th>SD + Met</th>
<th>HFD</th>
<th>HFD + Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST, µkat/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.7 ± 0.2 a</td>
<td>3.8 ± 0.1 b</td>
<td>4.1 ± 0.3 c</td>
<td>3.9 ± 0.5 a</td>
</tr>
<tr>
<td>I/R</td>
<td>7.0 ± 0.3 a</td>
<td>6.8 ± 0.3 b</td>
<td>12.6 ± 1.5 c,d</td>
<td>7.0 ± 0.8 a,d</td>
</tr>
<tr>
<td>ALT, µkat/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1 a</td>
<td>1.0 ± 0.1 f</td>
<td>0.7 ± 0.1 b</td>
</tr>
<tr>
<td>I/R</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>4.1 ± 0.8 a</td>
<td>2.0 ± 0.7 e,b</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE, n = 10. AST, aspartate aminotransferase; ALT, alanine aminotransferase; I/R, ischemia-reperfusion. Values marked with the same letter are statistically significantly different; 0.001; a,b,c,d,e,f,g P < 0.05; h,i,j,k,l,m,n,o P < 0.01.
showed in Fig. 5, after the addition of NADH to the SMP. We employed this method to investigate NADH-dependent ROS generation in vitro by SMPs prepared from HFD-fed groups. As shown in Fig. 5, after the addition of NADH to the SMP, we were able to detect the following free radicals: organic free radical OR, showing a symmetric singlet signal; nitroxide radicals NO: triplet spectrum 1:1:1; superoxide radical OOH: quartet 1:1:1:1; and hydroxyl radical OH: quartet 1:2:2:1, as adducts of these radicals with DMPO. The signal intensity was quantified by comparing the amplitude of the observed signal to the standard Mn²⁺ZnS and Cr³⁺MgO markers, the results of which are presented in Table 5. We found a significant increase in OH formation in animals subjected to I/R compared with sham-operated controls. A similar trend was observed for OOH, but it did not reach statistical significance (P = 0.061). Metformin treatment had no effect on sham-operated animals but completely prevented I/R-induced generation of OOH and OH radicals (P = 0.008 and 0.045, respectively). Other radical species were not affected by any of the interventions. In conclusion, our data indicate that long-term metformin administration in vivo effectively prevents I/R-associated ROS production from NADH-dependent substrates and succinate.

Metformin decreases reactive oxygen species formation from succinate and NADH, but not from glycerol-3-phosphate. Electron transport through the mitochondrial respiratory chain is associated with potential ROS generation. This risk is further exacerbated during reoxygenation after ischemia. In vitro studies identified three potential targets of the antioxidant effect of metformin: forward electron flux through complex I, reverse electron flux from complex II to complex I and mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH). In vitro, utilization of NADH as the sole substrate stimulates reverse electron flux. To characterize the combined effect of in vivo metformin treatment and HFD administration on ROS formation, we used a model of SMP. ROS production in vitro was measured by using a fluorescent probe DCFDA on three different substrates: NADH as a source of electrons for complex I, glycerol-3-phosphate (G-3-P) for mGPDH, and succinate for succinate dehydrogenase (Fig. 4). SMPS isolated from rats subjected to I/R without metformin treatment exhibited significantly increased ROS production from NADH and succinate (succinate >> NADH) when compared with sham-operated controls. Previous metformin administration reduced ROS production from both these sources. No effect of metformin was observed in animals that were not exposed to I/R. Rather surprisingly, we observed no effect of either I/R or metformin on ROS production from G-3-P.

EPR spectroscopy is the only technique that can directly detect and identify different types of free radicals. We employed this method to investigate NADH-dependent ROS generation in vitro by SMPS prepared from HFD-fed groups. As shown in Fig. 5, after the addition of NADH to the SMP
Significant effect in the SD group, but the drop in ATP concentration was further emphasized in the HFD/metformin group compared with animals fed only a HFD. ATP content reached only 60% of initial values after reperfusion and ATP repletion in the HFD/metformin group was also significantly slower than for both other groups. The HFD/metformin and HFD curves were significantly different \((P < 0.05)\) during the first 40 min of reperfusion \((t_{20} = t_{60})\).

Metformin attenuates the expression of proinflammatory markers in the liver. I/R injury is associated with the proinflammatory activation of immune cells in the liver, which is considered to be secondary to direct cellular damage resulting from ischemic insult. As we have shown, metformin reduced the I/R-induced liver injury. We further wanted to know whether this protection translated into an amelioration of proinflammatory status. Using RT-qPCR, we determined that the hepatic expression of proinflammatory markers TNF-\(\alpha\), TLR4, IL-1\(\beta\), and Ccr2 was significantly higher in the liver of HFD-fed animals subjected to I/R compared with sham-operated controls and that it was significantly attenuated by metformin treatment (Fig. 7). With the exception of TLR4, we did not find this I/R-induced proinflammatory activation in rats fed a standard diet. We did not observe any effect of the diet, metformin treatment, or I/R injury on the expression of alternative activation markers (Arg1, Mrc1, IL-10).

The liver accommodates two different subsets of macrophages: resident macrophages [Kupffer cells (KCs)] and macrophages that infiltrate the liver by circulation. The expression of markers characteristic of KCs (CD68, F4/80) was not different among the groups and did not depend on the diet or I/R injury. In contrast, the expression of Ly6c and CD11b, infiltrating monocyte and macrophage markers, respectively, were significantly increased in the liver of both SD+/I/R and HFD+/I/R groups. Metformin treatment significantly decreased...
activities in the liver of rats exposed to ischemia/reperfusion means were determined in the whole liver homogenate. Data are expressed as SOD, Catalase, GSH-Px, GSH, mM/mg protein.

Table 4. GSH concentration and antioxidant enzyme activities in the liver of rats exposed to ischemia/reperfusion

<table>
<thead>
<tr>
<th></th>
<th>SD</th>
<th>SD + Met</th>
<th>HFD</th>
<th>HFD + Met</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GSH, mM/mg protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7 ± 0.14±</td>
<td>6.8 ± 0.2</td>
<td>4.3 ± 0.1±</td>
<td>6.4 ± 0.1±</td>
</tr>
<tr>
<td>I/R</td>
<td>4.3 ± 0.4±</td>
<td>4.6 ± 0.3</td>
<td>1.7 ± 0.2±</td>
<td>3.5 ± 0.1±</td>
</tr>
<tr>
<td><strong>GSH-Px, µM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>540 ± 30±</td>
<td>583 ± 45</td>
<td>414 ± 22±</td>
<td>476 ± 40±</td>
</tr>
<tr>
<td>I/R</td>
<td>428 ± 22±</td>
<td>429 ± 25</td>
<td>258 ± 20±</td>
<td>435 ± 41±</td>
</tr>
<tr>
<td><strong>Catalase, µM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>758 ± 26±</td>
<td>820 ± 51</td>
<td>947 ± 13±</td>
<td>893 ± 30±</td>
</tr>
<tr>
<td>I/R</td>
<td>773 ± 33±</td>
<td>729 ± 49</td>
<td>508 ± 29±</td>
<td>764 ± 37±</td>
</tr>
<tr>
<td><strong>SOD, µU/mg protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>143 ± 10±</td>
<td>156 ± 5</td>
<td>91 ± 3±</td>
<td>129 ± 10±</td>
</tr>
<tr>
<td>I/R</td>
<td>95 ± 5±</td>
<td>138 ± 6±</td>
<td>73 ± 5±</td>
<td>130 ± 8±</td>
</tr>
</tbody>
</table>

GSH content was determined in the isolated mitochondria; all other parameters were determined in the whole liver homogenate. Data are expressed as means ± SE, n = 10. Values marked with the same letter are statistically significantly different; *p < 0.05; *p < 0.01; **p < 0.001.

Fig. 3. Mitochondrial respiration of NADH-dependent (A) and FADH-dependent (B) substrates. Liver mitochondria were isolated from rats fed HFD without or with metformin, half of the animals in each group (n = 5) being subjected to ischemia-reperfusion (I/R) as described in MATERIALS AND METHODS. The oxygen consumption rate of isolated liver mitochondria (10 µg protein per well) was monitored on an XF 24 Analyzer in the presence of chemicals added at time intervals shown on the graph. The additions included 5 mM malate (M), 10 mM glutamate (G), 4 mM ADP, 2 µM rotenone, and 10 mM succinate (final concentrations). Data are presented as means ± SE. The maximal respiration rate (M + G + ADP) was statistically significantly higher in HFD compared with all other groups.

DISCUSSION

Our data show that in vivo, metformin protects both the steatotic and normal liver from oxidative stress-related hepatotoxic injury caused by I/R. This effect is, at least in part, mediated by decreased mitochondrial ROS production resulting in reduced mitochondrial damage, attenuation of apoptotic/necrotic cell death, and elimination of inflammation.

It has been previously shown that several tissues endangered by oxidative imbalance associated with T2D, particularly the myocardium (38, 44), endothelial cells (5, 48), and the brain (13), could be protected by metformin administration. Surprisingly, there are scarce data regarding the antioxidant effect of metformin in the fatty liver, despite the fact that steatosis is a hallmark of T2D and that the fatty liver is highly susceptible to oxidative stress. One study conducted on 208 Indian diabetic patients showed that metformin restores antioxidant status in plasma (24). A hepatoprotective effect of metformin (decreased serum ALT and AST levels, improved histological parameters) was proved in two small studies focused on NASH patients (41, 65). Our results show that long-term metformin treatment not only alleviates chronic (HFD-induced) oxidative stress directly in liver tissue, but it also effectively protects the liver against acute and massive oxidative injury, particularly in the fatty liver. Nevertheless, the mechanism of metformin antioxidant activity is still far from clear.

The degree of oxidative stress results from the balance between the ROS formation rate and antioxidant defense capacity. Metformin is not able to scavenge superoxide radicals and is unlikely to engage in direct scavenging activity (6, 33, 49). In some models, antioxidative activity of metformin has been associated with its hypoglycemic effect, but in the presented study HFD-fed rats did not exhibit hyperglycemia. Numerous studies have demonstrated that both T1D (Type 1 diabetes) and T2D are associated with decreased activity and expression of antioxidant enzymes and that metformin treatment can restore normal conditions (13, 19, 62). In our experimental design, HFD-fed rats exhibited augmented markers of oxidative stress (lipoperoxides) accompanied by a significant decrease of liver GSH content and SOD activity and elevated catalase activity. I/R further exacerbated both oxidative injury and HFD-induced dysfunction of antioxidant mechanisms. Long-term metformin treatment normalized all these markers, both in sham-operated controls and in ischemic animals.

By directly measuring ROS production using a fluorometric assay and EPR, we showed that metformin attenuates mitochondrial ROS production. In reperfused liver, ROS can be generated by several mechanisms, including xanthine/xanthine oxidase, cytosolic NADPH oxidase, and the electron transport chain of mitochondria. Earlier work with rat hepatocytes using selective inhibitors has suggested that xanthine oxidase is the
main generator of ROS (1). However, xanthine oxidase inhibitors employed in these studies were later recognized as mitochondrial inhibitors as well and mitochondria are now considered to be an important source of ROS within hepatocytes (28). Estimating the relative contribution of mitochondria to total ROS production is difficult, because it depends on actual physiological conditions, such as the ratio of resting to phosphorylating mitochondria and the capacity of reverse electron transport. In the case of the ethanol-stressed liver, where nonmitochondrial ROS production from CYP2E1 is accentuated, mitochondrial contribution has been estimated at ~22–46% (30). In hypoxia/ischemia, when the portion of nonphosphorylating mitochondria is increased and reverse electron flow accentuated, this contribution may be significantly higher.

Several production sites for ROS are recognized at the respiratory chain, including complex I (36) and complex III (35), complex I being considered crucial for the regulation of mitochondria-related ROS production (66). At complex I, the superoxide radical may be generated either by reverse electron transfer (e− transport from succinate to NAD+ through complex I) upon succinate oxidation at complex II, or in lower concentrations. We are the first to demonstrate that metformin administered in vivo has the same effect, as we observed significantly decreased ROS production from succinate in submitochondrial particles isolated from metformin-treated rats subjected to I/R compared with untreated animals. Importantly, this effect persisted even when no metformin was added to the reaction in vitro. The contribution of reverse flux-related ROS generation under normoxic conditions remains questionable, but it seems to be highly relevant in hypoxia (30). This is in accordance with our results as we found the inhibitory metformin effect on ROS production from succinate only in rats that underwent I/R. Batandier et al. have shown that reverse flux-related ROS generation is very sensitive to changes in membrane potential. We have previously shown that biguanides decrease membrane potential as a consequence of mitochondrial complex I inhibition (16). We hypothesize that this phenomenon may, at least

### Table 5. Free radical generation in submitochondrial particles in vitro

<table>
<thead>
<tr>
<th></th>
<th>HFD</th>
<th>HFD + Met</th>
<th>HFD + I/R</th>
<th>HFD + Met + I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free organic radical</td>
<td>7.9 ± 1.1</td>
<td>7.9 ± 0.03</td>
<td>7.6 ± 0.8</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td>Nitrosyl radical NO·</td>
<td>3.8 ± 0.8</td>
<td>4.1 ± 0.2</td>
<td>4.3 ± 0.5</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>Superoxide radical OOH·</td>
<td>4.1 ± 0.2a</td>
<td>4.0 ± 0.25</td>
<td>5.1 ± 0.3ab</td>
<td>3.3 ± 0.4b</td>
</tr>
<tr>
<td>Hydroxyl radical OH·</td>
<td>1.2 ± 0.03c</td>
<td>1.1 ± 0.1</td>
<td>1.7 ± 0.24d</td>
<td>1.2 ± 0.05d</td>
</tr>
</tbody>
</table>

Submitochondrial particles prepared from liver mitochondria were isolated from rats fed HFD without or with metformin, half of the animals in each group being subjected to I/R as described in MATERIALS AND METHODS. Reactive oxygen species production in mitochondria was measured by electron paramagnetic resonance spectroscopy with 10 mM NADH as a substrate. Data are expressed as means ± SE, n = 5. Values marked with the same letter are statistically significantly different; abcdP < 0.05.

Fig. 4. Reactive oxygen species (ROS) production from different substrates in submitochondrial particles (SMPs). SMPs were prepared from liver mitochondria of rats fed HFD without or with metformin, half of the animals in each group being subjected to I/R as described in MATERIALS AND METHODS. ROS production in mitochondria was measured by DCFDA (10 μM) in the presence of either 10 mM NADH (A), 10 mM glycerol-3-P (B), or 10 mM succinate (C). Values represent fluorescence units and are normalized per 1 mg of mitochondrial protein. Data are presented as means ± SE, n = 5. Bars marked with the same letter are statistically significantly different; a,b,dP < 0.05; cP < 0.01.

Fig. 5. Typical electron paramagnetic resonance spectra obtained after addition of NADH (10 mM) to the suspension of SMP by using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin-trapping agent. The DMPO adducts were identified according to their g factor and DPPH served as an internal standard (g = 2.0036). OOH and OH signals were distinguished by detailed analysis of overlapping OOH and OH signals using computer simulation. OR, organic free radical; NO·, nitroxide radicals; OOH·, superoxide radical; OH·, hydroxyl radical; DPPH, diphenyl-1-pircylyldrazyl.
Fig. 6. Effect of long-term metformin administration on ATP resynthesis during reperfusion after partial ischemia. Partial ischemia was induced by portal vein ligation (time 0) and released 20 min later (time 20). The measurement continued in 5-min intervals. Liver ATP content was determined by $^{31}$P-NMR spectroscopy. Data are expressed as the relative change compared with the respective value in time 0 and presented as means ± SE, n = 10. The statistical significance of the course of the curves was tested by 2-way ANOVA with repeated measurements.

Partly, the beneficial effect of metformin on both forms of cell death as the consequence of lower flux-related ROS production. Inhibition of forward electron flux at the level of complex I by the specific inhibitor, rotenone, results in increased ROS production (46) and thus it can be assumed that metformin as a mild inhibitor of complex I may have the same effect. In contrast, we observed the inhibitory effect of metformin on NADH-dependent ROS production. This apparent contradiction could be reconciled in light of recent findings published by Matsuzaki and Humphries (43). Complex I has two reversible conformational states: active and deactivated (23). In its deactivated state, ROS production at complex I is significantly attenuated. Biguanides selectively inhibit the deactivated form of complex I. Consequently, this deactivation greatly enhances the sensitivity of complex I toward biguanides, forming a feedback loop that “locks” complex I in a deactivated state leading to decreased superoxide production capacity (43). Furthermore, ischemia represents a condition that promotes deactivation of complex I in vivo, thereby increasing the sensitivity to biguanide-mediated inhibition. On the basis of these findings, we suggest that metformin could serve as an antioxidant, particularly during oxidative stress associated with ischemia, and also following reperfusion.

Another potential source of ROS may be mGPDH, a key component of the glycerol phosphate shuttle, which ensures the transport of reduced equivalents from cytosol to complex II (45). Quite recently, mGPDH was recognized as a new target of metformin (40). Its contribution to ROS production has been documented in brown adipose tissue (65), but no data concerning liver mitochondria are available. In our study, the contribution of ROS produced by G-3-P was significantly lower than that from succinate. It also failed to respond to I/R and metformin. Given that mGPDH expression in the liver is very low (34), we do not consider this enzyme to be an important target of metformin antioxidant activity.

The accumulation of intracellular ROS induces cell death and, during hepatic I/R, hepatocytes undergo both apoptosis and necrosis (56). Some studies have suggested apoptosis to be the primary mode of death (55), whereas others have reported necrosis (61). In the present study, we found signs of both necroinflammation and ongoing apoptosis in the liver tissue of rats subjected to I/R, where the effect was more pronounced in the HFD group. We can explain the beneficial effect of metformin on both forms of cell death as the consequence of lower mitochondrial ROS production.

In the liver of metformin-treated animals, we observed lower expression of proinflammatory as well as infiltrating monocyte/macrophage markers 2 days after short-term ischemia, which points to the possible anti-inflammatory effect of metformin. The direct anti-inflammatory action of metformin has been reported in several animal experimental studies, both in the liver (69, 54, 47) and in circulating polymorphonuclear cells (9). In contrast to these experimental findings, metformin efficiency in human NASH treatment is the subject of open debate (58, 60). The beneficial effect of metformin in our study could be explained either by the direct effect of metformin on KCs or by the attenuation of intracellular ROS production in hepatocytes, resulting in diminished necrosis and suppressed inflammation.

Many reports demonstrated that ROS are important modulators of signaling pathways and the intensity of their production seems to be a key factor in discriminating between cell death and survival (53, 25, 39). It has been suggested that ROS act as second messengers that promote sustained activation of c-Jun NH$_2$-terminal kinase (JNK) and apoptotic signaling (59). Furthermore, they also have been shown to inhibit NF-κB by preventing transcription of survival genes (26). During reperfusion after ischemia, Kupffer cells generate ROS, which in turn activate JNK at least in part through the ROS-dependent AKS1 (apoptosis signal-regulating kinase 1) pathway and enhance secretion of various chemokines and cytokines including TNF-α (57). TNF-α may serve as a potent activator of either prosurvival or proapoptotic pathways (15), and some authors even report that it is a critical mediator in warm hepatic I/R injury (64). TNF-α released from Kupffer cells may activate TNF receptors on hepatocytes, which induce JNK activation as well as ROS production. JNK activation results in the activation of its downstream targets, i.e., AP-1 family members c-Jun, ATF-2, and JunD, which are involved in the regulation of inflammation, proliferation, and cell death (57). In addition, ROS oxidize and inactivate MAPK phosphatases, which dephosphorylate JNK, leading to its prolonged activation and augmentation of apoptosis (31). In the present study we showed data indicating that metformin reduces mitochondrial ROS production and we hypothesize that the attenuation of ROS signaling may explain, at least partly, the protective effect of metformin in IR. Another mechanism contributing to the reduction of inflammation in metformin-treated animals may be the reduction of ROS-induced lipoperoxide products formation during early reperfusion phase. These products are potent...
chemotactic factors for neutrophils and are the determining factor for the continuation of neutrophil recruitment and aggravation of injury (29). In accordance with this supposition we observed a significantly lower expression of infiltrating macrophages markers in metformin-treated animals subjected to IR.

Attenuation of mitochondrial respiration compromises the hepatocyte energy state. Foretz et al. (17) have shown that metformin decreases ATP content in a dose-dependent manner, both in primary hepatocytes and in the liver after metformin administration in vivo. Using direct 31P-NMR measurements in vivo, we demonstrated that long-term metformin treatment decreased ATP repletion during reperfusion, in terms of both rate and relative quantity. As we have previously shown (16), metformin does not function as an uncoupler and thus the decrease in ATP content reflects the weaker performance of the mitochondrial respiratory chain. Low ATP availability is generally an unfavorable condition and may contribute to high-dose metformin toxicity, but we suggest that under conditions of acute oxidative stress the beneficial consequences of diminished electron flux through the respiratory chain, i.e., lower ROS formation, outweighs the disadvantages of compromised energy production.

In conclusion, we demonstrate that metformin protects the fatty liver from acute oxidative stress-related mitochondrial injury and cell death. We propose that the beneficial effect of metformin action is based on the combination of three contributory mechanisms: increase of antioxidant enzyme activity,
lower mitochondrial ROS production at complex I, and reduction of postschismic inflammation. The presented data support the extension of the therapeutic application of metformin as an antioxidant, particularly during IR-related oxidative stress.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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