Ischemic pre- and postconditioning has pronounced effects on gene expression profiles in the rat liver after ischemia/reperfusion

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Knudsen AR, Kannerup AS, Dich R, Funch-Jensen P, Grønbæk H, Kruhøffer M, Mortensen FV. Ischemic pre- and postconditioning has pronounced effects on gene expression profiles in the rat liver after ischemia/reperfusion. Am J Physiol Gastrointest Liver Physiol 303: G482–G489, 2012. First published June 7, 2012; doi:10.1152/ajpgi.00337.2011.—Ischemic pre (IPC) and postconditioning (IPO) protect the liver against ischemia/reperfusion injuries (IRI). Conditioning involves several different trigger factors, mediators, and effectors, many of which are affected during the early phase of reperfusion, ultimately resulting in decreased liver injuries. The aim of the present study was to investigate the genomic response induced by IPC and IPO in ischemia/reperfusion-damaged rat liver biopsies. Forty-eight male Wistar rats were divided into five groups: sham (n = 8), IRI (n = 10), IPC (n = 10), IPO (n = 10), and IPC + IPO (n = 10). The rat livers were subjected to 30 min of ischemia. Liver biopsies and blood samples were taken after 30 min of reperfusion. The biopsies were analyzed using cDNA microarrays with validation by quantitative RT-PCR. The significance analysis of microarray was used to identify genes with changed expression levels. A comparison analysis of the intervention groups showed a highly increased number of genes, with significantly different expression in the conditioned groups compared with the IRI group. A total of 172 genes were identified as the most highly affected, and these genes showed similar patterns with regard to the up- and downregulated expression levels within the conditioned groups. Pathway analysis of the 172 genes identified four networks that were involved in increased gene expression, cellular growth, and proliferation. In conclusion, the present study demonstrated that IPC, IPO, and IPC + IPO had pronounced effects on the expression levels of a large number of genes during early reperfusion. IPC, IPO, and IPC + IPO seem to mediate their protective effects by regulating the same genes and genetic networks. These identified networks are known to be involved in maintaining cellular homeostasis.

ischemia/reperfusion injuries; liver failure; microarray analysis

DURING HEPATIC RESECTION, vascular clamping is a frequently used method to control blood loss (35). Prolonged periods of tissue oxygen deprivation convert cellular metabolism to anaerobic pathways. Reperfusion, with the restoration of blood and oxygen delivery to the liver, results in hepatocellular injuries (14). Ischemia/reperfusion injuries (I/R injuries) are inevitable after periods of ischemia. The extent of the hepatic damage is determined by multiple factors, including the duration of the ischemic period, presence of preexisting hepatic diseases, and usage of hepatoprotective methods, such as ischemic conditioning. The early phase of reperfusion seems to be crucial in the pathogenesis of I/R injuries. During this phase, the ischemic insult is aggravated by the formation of reactive oxygen species, mainly from Kupffer cells (14). Further damage is caused by an excessive inflammatory response and the activation of macrophages, neutrophils, and lymphocytes during the late phase (13, 14).

Ischemic pre- and postconditioning (IPC and IPO, respectively), which are defined as brief periods of ischemia and reperfusion before or after sustained ischemia, have been proven to reduce the extent of the I/R injuries in the liver (3, 5, 7, 21, 27, 32, 36, 37). The mechanism responsible for this endogenous hepatoprotection is only partly known. It involves several different trigger factors, mediators, and effectors, many of which are affected during the early phase of reperfusion, ultimately resulting in decreased liver injuries.

The use of DNA microarrays enables us to examine the expression levels of all ~29,000 rat genes, potentially uncovering genes and pathways affected by ischemic conditioning. Gene expression levels have been investigated after I/R injuries of the liver (12, 15, 23). Previously, we (16, 17) showed that IPC and IPO induce genes involved in angiogenesis. However, the effects of IPC and IPO on the overall transcriptional response to I/R injuries have not yet been investigated and compared in the same study.

The aim of the present study was to investigate the genomic response induced by IPC and IPO in I/R-damaged liver biopsies using microarray analysis. By using this method, we wanted to identify key molecules and pathways that were significantly affected by ischemic conditioning during the early phase of reperfusion.

MATERIALS AND METHODS

Experimental design. The surgical and experimental protocols were approved by the Danish Research Animal Committee, Copenhagen, Denmark, under license number 2007/561-1311, and followed the rules of Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

A total of 48 male Wistar rats, weighing 300–350 g (M&B Taconic, Eiby, Denmark), were used for the experiment. The animals were housed in standard animal laboratories with the temperature maintained at 23°C, an artificial 12-h light-dark cycle, and free access to food and water. The rats were randomly divided into five groups as follows: sham (n = 8), IRI (n = 10), IPC (n = 10), IPO (n = 10), and IPC + IPO (n = 10). All animals were anesthetized using 0.75 ml/kg hypnorm sc (fentanyl/fluanisone; Jansen Pharma, Birkeroed, Denmark) and 4 mg/kg midazolam sc (dormicum; La Roche, Basel, Switzerland) and placed on a heated pad. A midline laparotomy was performed, and total hepatic ischemia was accomplished using a

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microvascular clamp placed on the hepatoduodenal ligament. Reflow was initiated after 30 min of ischemia by removal of the clamp. Discoloration of the liver was used as a positive marker for hepatic ischemia. Reperfusion was ascertained by a return of the normal reddish color of the liver.

Sham-operated rats were subjected to a laparotomy and immobilization of the hepatoduodenal ligament without clamping of the vascular structures. The rats in the remaining groups were subjected to 30 min of total liver ischemia, followed by 30 min of reperfusion before the liver biopsies were taken. The IPC consisted of 10 min of liver ischemia followed by 10 min of reperfusion. The IPO consisted of three cycles of 30 s of reperfusion and 30 s of ischemia applied immediately after the 30 min of total liver ischemia. In the IPC + IPO group, both interventions were performed (Fig. 1).

The liver biopsies were sampled from the right lobe, immediately snap frozen in liquid nitrogen, and stored at ~80°C for further analysis. Blood samples were taken from the common iliac artery for the measurements of alanine aminotransferase (ALT), alkaline phosphatase, and bilirubin. All rats were then euthanized with an overdose of pentobarbital.

RNA isolation. The liver biopsies were homogenized in RLT plus buffer on a Tissuelyser (Qiagen, Hilden, Germany). Total RNA was extracted on a QIAsymphony SP (Qiagen) using the QIAsymphony RNA extraction kit. The same batch of total RNA was used both for the microarray analysis and quantitative real-time PCR validation.

Microarray analysis. The gene expression analyses were performed according to the Affymetrix (Santa Clara, CA) standard procedures. In brief, 150 ng of total RNA were used as the target preparation in the starting material for the cDNA synthesis kit and wild-type terminal labeling kit. The target was loaded onto the Affymetrix Rat Exon 1.0 ST array cartridge and hybridized for 16 h. The arrays were washed and stained in the Affymetrix Fluidics Station preparation in the starting material for the cDNA synthesis kit and the microarray analysis and quantitative real-time PCR validation.

Quantitative real-time PCR. To validate the microarray expression data, quantitative real-time PCR (qRT-PCR) was performed using the Fluidigm BioMark and ABI 7900HT systems. The expression levels of 12 key genes of interest were analyzed using rat-specific TaqMan assays (ABI, PN4448892) and two rat-specific endogenous control assays (ABI, PN4352338E and PN4352340E). A list of the TaqMan assays is given in Table 1. The 12 genes of interest were analyzed using one Fluidigm 96.96 Dynamic array with assay triplicates. The setup was in accordance with the manufacturer’s protocol. A total of 100 ng of RNA was used as the input in the 20-µl reverse transcription reaction. The reverse transcription was performed using the high capacity cDNA reverse transcription kit (ABI, PN4368813) in accordance with the manufacturer’s protocol. The expression levels of the normalization genes were analyzed using the ABI 7900HT. The PCR amplification was performed in double 10-µl reactions using 10 ng of input RNA. The amplification was performed using the standard conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The amount of mRNA was normalized to the geometric mean of the two stably expressed rat normalization genes GAPDH and β-actin.

Statistical analyses. The statistical analyses of the blood samples were performed using SPSS11.0 (SPSS, Chicago, IL). The data analyses were performed using the nonparametric Kruskal-Wallis (ANOVA) test, followed by the Mann-Whitney U-test. A P value <0.05 was considered significant.

I P C   Group
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Table 1. List of rat-specific TaqMan assays used for qRT-PCR validation (ABI, N4448892)

<table>
<thead>
<tr>
<th>Assay</th>
<th>TaqMan Assay</th>
<th>Gene Name</th>
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<tbody>
<tr>
<td>1</td>
<td>Rn00568504_m1</td>
<td>BTG family. member 2</td>
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<tr>
<td>2</td>
<td>Rn02396759_m1</td>
<td>FBJ osteosarcoma oncogene</td>
</tr>
<tr>
<td>3</td>
<td>Rn00561138_m1</td>
<td>Early growth response 1</td>
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<tr>
<td>4</td>
<td>Rn00589521_m1</td>
<td>Suppressor of cytokine signaling 2</td>
</tr>
<tr>
<td>5</td>
<td>Rn00590440_m1</td>
<td>Caspase 12</td>
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<tr>
<td>6</td>
<td>Rn00572991_s1</td>
<td>Jun oncogene</td>
</tr>
<tr>
<td>7</td>
<td>Rn00710306_m1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>8</td>
<td>Rn00561416_m1</td>
<td>Insulin-like growth factor binding protein 3</td>
</tr>
<tr>
<td>9</td>
<td>Rn00565713_m1</td>
<td>Insulin-like growth factor binding protein 1</td>
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<tr>
<td>10</td>
<td>Rn00561507_m1</td>
<td>Myelocytomatisis oncogene</td>
</tr>
<tr>
<td>11</td>
<td>Rn00563784_m1</td>
<td>Activating transcription factor 3</td>
</tr>
<tr>
<td>12</td>
<td>Rn00567697_m1</td>
<td>Adenosine A2B receptor</td>
</tr>
<tr>
<td>13</td>
<td>RatGAPDH/435238E</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>14</td>
<td>RatACTB/4352340E</td>
<td>Actin-β</td>
</tr>
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Additional pathway analysis was performed using the Ingenuity Pathway Analysis (IPA) 8.6 application (Ingenuity Systems, Redwood City, CA).
This analytic tool can uncover canonical signaling pathways and gene networks within an uploaded gene list.

RESULTS

Liver parameters. The ALT levels increased in the IRI, IPC, IPO, and IPC + IPO groups compared with the sham group (P = 0.000). Furthermore, ALT was significantly elevated in the IPC + IPO group compared with the IRI (P = 0.043) and IPC (P = 0.015) groups. No difference was found between the IPO and the IPC + IPO groups (Fig. 2). The alkaline phosphatase and bilirubin levels were both comparable among all groups.

Unsupervised two-class unpaired SAM. The data reduction (SD/mean > 0.022) selected the 6,392 most varying genes. The two-class unpaired analysis of the intervention groups vs. the sham group identified genes within these four groups that were significantly affected (Fig. 3). A large number of genes were significantly affected by ischemic conditioning, especially in the IPO and IPC + IPO groups (Fig. 2). A comparison analysis between the IPC and IPO groups identified 10 differentially expressed genes.

Unsupervised significance analysis for SAM multiclass analysis. The data reduction was carried out by removing genes with SD/mean < 0.1, which identified the 2,203 genes with the largest variations among the groups. This increased threshold was used to identify the most differentially expressed genes in the following analysis. The SAM multiclass analysis identified 172 differentially expressed genes. The mean expression values of the 10 samples in each of the 5 groups (8 in the sham group) were used to create a hierarchical cluster of the 5 study groups (Fig. 4). The three groups subjected to ischemic conditioning formed a cluster that was easily distinguishable from the cluster formed by the sham and IRI groups. All 172 significant genes are listed in Supplemental Table 1 (Supplemental Material for this article is available online at the Am J Physiol Gastrointest Liver Physiol website).

A DAVID functional cluster analysis of the differentially expressed genes revealed that the largest functional cluster of genes included in the analysis was DNA binding and transcription (23%), followed by cellular membrane (13%), apoptosis (13%), and metabolic process (12%; Fig. 5). IPA was used to identify the molecular pathways associated with the differentially expressed genes. We uncovered four highly significant networks (Fig. 6). In general, these networks are all known to stimulate gene expression, cellular growth, and proliferation.

Quantitative validation of microarray data. Validation of the microarray data was performed using qRT-PCR. We selected 12 key regulated genes to validate in all 48 liver samples. The genes were selected as follows: 10 genes that were found to be upregulated in the conditioned groups and 2 genes that were found to be normo-regulated. The results are listed in Table 2. We found a high agreement between the two methods; thus, the qRT-PCR confirmed our microarray data.

DISCUSSION

Ischemic conditioning has previously been shown to protect the liver against I/R injuries (3, 5, 7, 21, 27, 32, 36, 37). This study was designed to detect gene expression differences during the early moments of reperfusion. We chose to measure the gene expression levels at 30 min of reperfusion because the early moments of reperfusion seem to be essential in the pathogenesis of I/R injuries (8). We demonstrated that ischemic conditioning had a pronounced effect on the number of genes that were up- and downregulated during the early phases of reperfusion (Fig. 3). The large number of upregulated genes in the IPC group is in accordance with previous reports involving the intestines (23), heart (20, 31), leukocytes (19), and liver (24). No studies exist on the effect of IPO and IPC + IPO on gene expression.

Circulating ALT levels were significantly elevated in the four intervention groups compared with the sham group. Given that ALT has been suggested as a marker of hepatocellular injury (30), this result indicates that our model did induce hepatocellular damage. The ALT levels were not significantly reduced by IPC or IPO as previously described (3, 21, 36). In a new study, however, we showed that both IPC and IPO reduce the amount of necrotic tissue in the liver by 30% after 24 h of reperfusion (18). In this study, we did also not find any difference in the ALT levels among the IRI, IPC, and IPO groups in the early and late reperfusion periods.
To identify key genes that are regulated during the early phase of reperfusion, we enhanced the selection criteria by applying a multiclass analysis, which revealed 172 differentially expressed genes. A hierarchical cluster analysis of these 172 genes showed 2 groupings, one comprising the 3 conditioned groups and the other comprising the IRI and sham groups. This result demonstrates that the significant genes are affected by ischemic conditioning and led us to the following two conclusions. First, IPC and IPO affect the expression of the same genes, and these genes have similar up- or downregulatory patterns, indicating that the mechanisms behind their protective effects are, to a large extent, identical. This observation is supported by the identification of only 10 differentially expressed genes when the IPC and IPO groups were compared in a two-class comparison analysis. Second, a large number of genes are affected, indicating that the protective properties of conditioning are likely mediated by several different cellular pathways. Finally, a group of 20 genes was less expressed in the IRI-group than in the sham-group, as seen in Fig. 4, although more expressed than in the conditioned groups. The significance of this finding is unclear, since the functions of these genes are still unknown.

Cluster and network analysis. A DAVID annotation analysis divided the 172 significantly differentially expressed genes into

![Hierarchical cluster analysis of the 5 study groups using 172 significant genes. Red indicates an increased expression level, whereas green indicates a decreased expression level. Expression levels of each gene are calculated as the mean of the group.](image1)

![DAVID functional annotation cluster analysis. Analysis was performed on the 172 significantly differentially expressed genes. Chart describes the gene ontology terms associated with this gene list and the number of genes associated with each term. Terms with 5 or less associated genes are compiled into Others. Several genes are represented in more than one cluster.](image2)
14 functional clusters (Fig. 5). The largest cluster was related to DNA binding and transcription, the second largest to cellular membrane function and the third largest to apoptosis. This result indicates that ischemic conditioning, whether IPC or IPO, mediates its protective effects by activating the expression of genes crucial to cellular homeostasis. This finding was further supported by the IPA analysis, which identified four highly significant pathways that all affected the cell cycle, growth, and proliferation.

In network 1 (Fig. 6A), 18 genes (focus genes) were identified as part of our data, with 17 being upregulated and 1 downregulated, when the mean expression levels of the conditioned groups were compared with the mean expression levels of the IRI and sham groups. Several transcription factors, such as Jun, Junb, Egr1, Fos, Atf3, Klf6, and Trib1, were overexpressed in the conditioned groups and are discussed in further detail below. Gdf15 and Fgf21, which are growth factors, were upregulated in the conditioned groups. The anti-apoptosis genes Socs2 and Btg2 were upregulated, whereas Ubd, which is involved in protein degradation and has a proapoptotic function, was downregulated in the conditioned groups. Rhob and Nr4a1, which function as regulators of apoptosis, the cell cycle, and gene expression, were upregulated. Myd116, which is involved in the cellular response to stress, was induced in the conditioned groups. Rnd3 is a cellular transmitter and is upregulated in the conditioned groups. Hspb1, which was upregulated in the conditioned groups, is an intracellular anti-inflammatory mediator and increases the cellular redox defense.

Network 2 (Fig. 6B) encompassed 15 focus genes, with 13 being upregulated in the conditioned groups. The upregulated genes included the following: Adora2b, an adenosine receptor previously suggested to confer protection against I/R injuries (26, 38); Klf6, which regulates transcription factor activity;
Rnd3, which is involved in signal transmission; Orc4l, which affects DNA replication; Okl38, which is a cellular growth inhibitor and involved in the regulation of apoptosis; and Phlda3, which is an inducer of apoptosis.

Network 3 (Fig. 6C) encompassed 13 focus genes, with 11 being upregulated in the conditioned groups. These upregulated genes were the following: Gadd45a and Gadd45g, which are stress-induced regulators of DNA-repair and cell cycle

Table 2. Comparison between microarray data and quantitative RT-PCR analysis of 12 selected genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>IRI</th>
<th>IPC</th>
<th>IPO</th>
<th>IPC + IPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTG family member 2</td>
<td>2.25</td>
<td>4.03</td>
<td>5.65</td>
<td>4.07</td>
</tr>
<tr>
<td>FBJ osteosarcoma oncogene</td>
<td>1.51</td>
<td>2.67</td>
<td>2.59</td>
<td>2.92</td>
</tr>
<tr>
<td>Early growth response 1</td>
<td>1.84</td>
<td>6.36</td>
<td>4.43</td>
<td>2.95</td>
</tr>
<tr>
<td>Suppressor of cytokine signaling 2</td>
<td>2.51</td>
<td>3.73</td>
<td>4.66</td>
<td>4.31</td>
</tr>
<tr>
<td>Caspase 12</td>
<td>1.40</td>
<td>1.79</td>
<td>1.87</td>
<td>2.24</td>
</tr>
<tr>
<td>Jun oncogene</td>
<td>2.04</td>
<td>2.91</td>
<td>4.15</td>
<td>3.52</td>
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<tr>
<td>Insulin-like growth factor 1</td>
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<td>0.91</td>
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<tr>
<td>Insulin-like growth factor protein 3</td>
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<td>0.79</td>
<td>0.89</td>
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<tr>
<td>Insulin-like growth factor binding protein 1</td>
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<td>1.76</td>
<td>3.49</td>
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<td>Myelocytomatosis oncogene</td>
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<td>Activating transcription factor 3</td>
<td>2.99</td>
<td>5.97</td>
<td>7.59</td>
<td>6.18</td>
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<tr>
<td>Adenosine A2B receptor</td>
<td>1.43</td>
<td>0.27</td>
<td>1.70</td>
<td>2.44</td>
</tr>
</tbody>
</table>

Values indicate mean fold change in gene expression levels compared to the sham group. IRI, ischemia/reperfusion injuries; IPC and IPO, ischemic pre- and postconditioning; MA, microarray data.

Fig. 7. Bar charts of microarray vs. quantitative (q)RT-PCR. A high degree of correlation was found in the expression levels of 6 immediate early genes differentially regulated during ischemia/reperfusion as determined by the microarray and qRT-PCR analyses. Solid bars indicate microarray data, whereas the open bars indicate data from the qRT-PCR validation analysis. Bars indicate the fold change in the gene expression levels compared with the sham group after 30 min of reperfusion. Jun, c-jun; Btg2, B-cell translocation gene 2; Fos, FBJ osteosarcoma oncogene; Myc, myelocytomatosis oncogene; Egr1, early growth response 1; Atf3, activating transcription factor 3.
control; Casp12, which is an apoptotic inducer; Cxcl2, which is a chemokine and is involved in the cellular response to inflammation; and Cish, which affects cell growth and serves as a negative regulator of signal transduction.

**Network 4** (Fig. 6D) comprised six focus genes, with five being upregulated in the conditioned groups. Angptl4 is an anti-apoptotic and increases angiogenesis. Apold1 increases angiogenesis, and Igfbp1 induces cell growth and tissue regeneration.

Alone and in combination, these pathways seem to increase the cellular resistance to stressful conditions and consequently also increase the resistance of the rat liver to I/R injuries.

**Immediate early response genes and cell proliferation.** The immediate early genes are defined as genes activated rapidly and transiently by several cellular stimuli, including ischemia/reperfusion. Most of these genes function as transcription factors and are early regulators of cell proliferation. Several genes in this group were identified in the present study as key regulated genes. Btg2, an anti-apoptotic gene, has been identified in other expression studies as a key gene regulated by IPC (2, 31). This finding is in agreement with our study, in which Btg2 was upregulated 5.7-fold in the IPC group and 4.1-fold in the IPO group, compared with 2.3 fold in the IRI group (Fig. 7). The early growth response gene Egr1 was also upregulated by conditioning, especially by IPC. The same effect of IPC has also been reported in a study on rat spinal cords (2) at 30 min of reperfusion. In a study on mice hearts, Egr1 was unaffected between the conditioned and control groups after 15 min of ischemia (20). This result, however, might be explained by the transient regulation of these genes. Egr1 is a transcription factor that appears to be important for cellular differentiation and proliferation. Myc, similar to Fos, Jun and Atf3 (see below), is a transcription factor. Myc and Fos have previously been shown to be induced by ischemic conditioning (25). Myc was also induced in the IPC group in our study, whereas the expression levels were similar in the IPO and IRI groups. Jun, Fos and Atf3 are functionally related genes that induce the transcription of several genes containing activating protein 1 (AP-1) binding sites (10). These sites can activate cellular proliferation, differentiation and apoptosis. The coexpression of Jun and Fos has been suggested to mediate early tissue repair in the liver (29). Furthermore, Fos seems to induce cell cycle re-entry (1). Both Jun and Fos were induced by IPC and IPO in this study (Fig. 7). The expression of Atf3 has been induced by IPC in the heart and spinal cord (2, 31). We also found the expression of this gene to be highly induced by both IPC and IPO.

Overall, our data suggest that both IPC and IPO induce basic cellular pathways, leading to the activation of tissue repair and cellular proliferation pathways. This observation is in agreement with a previous report (33). Cardiac research has shown that protection during the early phases of reperfusion is dependent on the posttranslational modification of preexisting proteins, whereas protection during the late phase is dependent on altered gene expression and de novo protein synthesis (4, 22). We suggest that the induction of immediate early genes by IPC and IPO is crucial in this context and ultimately reduces I/R injuries in the liver.

The combination of IPC and IPO has been speculated to offer an additive protective effect on the liver with regard to I/R injuries (9, 37). However, we showed that the gene expression profile in the IPC + IPO group is very similar to that of the IPC and IPO groups, indicating a similar, but not additive, effect. Given that the previous studies (9, 37) did not find any additive effects with regard to I/R injuries, we conclude that this combined approach is not especially effective.

A limitation of this study is the assumption that the transcriptional profiles mirror the changes in the protein expression levels. Posttranslational modifications of proteins induced by I/R and ischemic conditioning are not detected by this method. However, the transcriptional profile can be assumed to ultimately be modulated by the corresponding proteomic response, which is representative of the overall molecular response to I/R and ischemic conditioning.

We performed RNA extraction from liver biopsies containing hepatocytes, endothelial cells, immune cells, and others, which prevents the ability to distinguish the differences in the transcriptional response among these different cell types. Because hepatocytes make up the largest portion of these cells, we assume that our results represent the transcriptional response in these cells.

**Conclusion.** The present study demonstrated that IPC, IPO, and IPC + IPO had pronounced effects on the expression levels of many genes, especially the immediate early gene respond seems to be important. To a large extent, IPC, IPO, and IPC + IPO seem to mediate their protective effects by regulating the same genes. Further pathway analysis of these genes identified the upregulation of gene networks involved in cellular homeostasis and cell proliferation.

**GRANTS**

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


