Ischemic preconditioning alters the pattern of gene expression changes in response to full retinal ischemia

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Purpose: Ischemic conditions in the retina have been implicated in several retinopathological conditions. Experimentally induced ischemia for 60 min followed by reperfusion leads to a loss of neurons in the inner retina. In contrast, a 5 min ischemic episode triggers a series of alterations that protect the retina against the damaging effects of a subsequent 60 min ischemic insult. This phenomenon is called ischemic preconditioning (IPC). To study the changes altered by IPC, we assessed the gene expression patterns in the rat retina after ischemia (60 min) followed by reperfusion (I/R) and compared these to the gene expression patterns after ischemia/reperfusion in preconditioned animals (IPC-I/R).

Methods: Changes in gene expression were studied, by means of microarrays, at 1, 2, 6, and 12 h after I/R in naïve and preconditioned animals. To identify functional pathways of interest, we used significantly regulated genes as input for gene ontology analysis. Microarray results were validated by real-time quantitative PCR.

Results: Most genes that were altered by I/R showed a comparable change in both naïve and preconditioned animals. Differential expression was found for a total of 1312 genes of the 20,280 features (6.4%) present on the array with a differential change of 1.7 fold or more. The list of genes with a differential change was characterized by a statistically significant overrepresentation of genes associated to the gene ontology terms tRNA aminoacylation (with a decreased expression due to preconditioning), immune response (with most genes upregulated), and apoptosis (mixed direction of changes). The results of quantitative PCR assays were in agreement with the microarray data.

Conclusions: The response of several functional groups of genes on ischemia was altered by a preconditioning stimulus. Most prominent differences were found for the group of genes encoding for aminoacyl-tRNA synthetases (ARSs), which is in line with the previously observed decreased expression of ARSs after induction of preconditioning. Our observations indicate that activation of translational activity may be a mediator of ischemia-associated damage in the retina, and IPC may prevent activation of this mechanism. An altered expression of genes implicated in immune response and in apoptosis may also be involved in effectuating IPC.

Retinal ischemia for a duration of 60 min leads to a selective loss of amacrine and ganglion cells via apoptosis [1-3]. Ischemia is one of the factors implicated in glaucoma-associated cell loss in the inner retina. Laser Doppler flow cytometry showed that optic nerve blood flow is already decreased in glaucoma suspects before visual field defects become manifest, suggesting an involvement of ischemic conditions in the early stages of glaucoma [4]. Glaucomatous visual field defects have been reported to occur prior to structural changes of the optic nerve head or nerve fiber layer, and these typical structural aberrations may be a consequence of the loss of nerve fibers caused by retinal ischemia [5-9]. The loss of retinal ganglion cells in the glaucomatous retina corresponds to the pattern of neuronal degeneration, by apoptosis, after experimental ischemia [1,3]. However, a 5 min episode of retinal ischemia, does not inflict damage but rather triggers an endogenous form of neuroprotection, termed ischemic preconditioning (IPC) or tolerance. The retina of preconditioned animals is fully protected against the morphological and functional damage resulting from a full ischemic insult [10,11]. Insight in the underlying mechanisms of IPC may lead to a better understanding of how retinal cells deal with stressful conditions, mechanisms that may falter in retinopathological conditions. IPC induced protection is only transient with maximum protection achieved 24-72 h after the preconditioning stimulus. Although IPC has also been observed in brain, spinal cord, and other tissues, the protective effect of IPC in the retina is particularly robust because it offers full protection against functional impairment and cell loss while in other tissues cell loss is only partially prevented [12-15]. The involvement of Hif1 transcription factor [13,16], Hsp27 (Hspb1) upregulation [16], stimulation of adenosine A1 and A2α receptors [17,18], protein kinase C activation [17], leukocyte rolling inhibition [19], inducible nitric oxide synthase [20], and the opening of mitochondrial potassium-dependent ATP channels [21] have all been proposed as underlying mechanisms of preconditioning (for reviews see [10,22]).

In a previous study, we used a microarray approach to describe the alterations in retinal gene expression as a result of the induction of IPC [23]. Changes were first dominated by altered expression levels of genes encoding transcription factors. At stages coinciding with the time window of effective protection, transcript levels of aminoacyl-tRNA synthetases...
Animals and anesthetics: Animal handling and experimental procedures were reviewed and approved by the ethical committee for animal care and use of the Royal Netherlands Academy for Sciences, acting in accordance with the European Community Council directive of 24 November 1986 (86/609/EEC) and the ARVO statement for the use of animals in ophthalmic and vision research. The procedure to induce transient ischemia followed by reperfusion (ischemia/reperfusion: I/R) has been described in detail previously [24-27]. In short, adult male Wistar rats (Harlan Laboratories, Horst, the Netherlands) weighing 200-300 g were anesthetized by an intramuscular injection of hypnorm (fentanylcitrate and fluanisone; 0.5 ml/kg body weight; Jansen Pharmaceuticals, Beerse, Belgium) in combination with an intraperitoneal injection of valium (diazepam; 0.5 ml/kg body weight; Hoffman-La Roche, Basel, Switzerland). Neither hypnorn nor valium is known to have neuroprotective properties. A local anesthetic, Oxybuprocain (benzalkoniumchloride; 0.4% w/v; Smith and Nephew, Hoofddorp, the Netherlands) was applied to both eyes and mounted onto a stereotactic frame (Stoelting, Wood Dale, IL). A steel 30-gauge infusion needle (BD Biosciences, Alphen aan den Rijn, The Netherlands) connected to a saline reservoir was placed in the middle of the anterior chamber of the left eye. The other eye served as control and was either not-operated on or was sham-operated on by inserting a needle into the anterior chamber without elevating intraocular pressure. The reservoir was opened and lifted to 1.70 m. After 60 min of ischemia, the reservoir was lowered and reperfusion resumed immediately.

The first group of animals (I/R), was subjected to 60 min ischemia followed by reperfusion. The retinas were studied after the following reperfusion times: 1 h (n=5), 2 h (n=5), 6 h (n=4), and 12 h (n=4). The second group (IPC-I/R) was preconditioned by 5 min of ischemia followed by a reperfusion interval of 24 h, and then allowed to undergo a second period of ischemia lasting 60 min. Retinas were studied after the following reperfusion times: 1 h (n=7), 2 h (n=6), 6 h (n=6), and 12 h (n=7). This protocol leads to full protection against the deleterious effects of 60 min ischemia has been shown previously by histology [23]. Animals were killed by the administration of an intraperitoneal overdose of sodium pentobarbital (0.8 ml; 60 mg/ml, Ceva Santa Animale, Maassluis, The Netherlands).

RNA isolation: The isolation of total RNA from the retina has been described in detail [23,28]. In short, the whole retina free of vitreous was isolated, frozen on dry ice, and stored at -80 °C. Frozen retinas were thawed in Trizol (Invitrogen, Breda, The Netherlands) and homogenized immediately. Total RNA was isolated following the manufacturer’s instructions. Precipitated RNA was dissolved in 8 µl RNase-free water. The RNA yield was around 10 µg retina (ND-1000 spectrophotometer; NanoDrop Technologies, Wilmington, DE) and quality checks showed sharp ribosomal RNA bands with minimal degradation (2100 Bioanalyser, Agilent Technologies, Amstelveen, The Netherlands).

Experimental set-up and probe generation: Of each animal, 1 µg total RNA from each sample was used to make pooled samples for each experimental group (n=4-7/group) and one pooled sample for all contralateral control retinas (n=44) including 34 unoperated eyes and 10 sham-operated eyes. To test the validity of pooling the non-operated and sham-operated samples for microarray analysis, we determined by qPCR the transcript levels of reference genes and a selection of stress-induced genes (c-fos, c-jun, Il6, Hmx1, and Gfap). No differences were found between the unoperated and sham-operated retinas, which is in line with our previous findings [27,28]. Moreover, the statistical analysis of the complete quantitative PCR (qPCR) dataset for 41 different genes revealed no differences between the unoperated and sham-operated retinas.

Details of the amino allyl-UTP aRNA probe synthesis were described previously (Amino Allyl MessageAmp aRNA kit, Ambion, Nieuwerkerk a/d IJssel, The Netherlands) [23,29]. In short, or first strand cDNA synthesis, 1.5 µg RNA of the pooled sample was primed with a T7 Oligo(dT) primer. Following second strand cDNA synthesis, in vitro transcription generated amino allyl-UTP labeled aRNA. Electropherograms of the amplified aRNA showed a symmetrical length distribution with a peak around 1.5 kb with a maximum length of 5.5 kb and no differences in size distribution between the different samples. The aRNA was coupled to Cy3 or Cy5 monoreactive dyes (Amersham and purified from excess dye over a Chromaspin-30 column (Clontech). Incorporation efficiency and yield were determined on a spectrophotometer (NanoDrop Technologies). The aRNA was labeled with Cy3 or Cy5 monoreactive dyes (Amersham, Eindhoven, The Netherlands) and purified. A common reference design was used for microarray hybridization. A 30 µg sample of aRNA of the control-group was labeled with Cy3. A 1 µg aliquot served as the identical common reference present on all arrays and was hybridized simultaneously with 1 µg Cy5-labeled aRNA from one of the experimental groups.

Microarrays: Oligonucleotide arrays were obtained from Agilent (22K catalog rat array, 60-mer oligonucleotides, product number G4130A). The complete design can be found at Agilent or GEO-NCBI. These arrays are designed for the simultaneous hybridization of two aRNA samples labeled with
Cy5 (treatment-group) and Cy3 (control). After normalization, the dye ratio represented the transcript ratio. The details of this technique have been described before [23,30], and can be found at Agilent.

Data analysis: The array images were acquired using an Agilent microarray scanner set at 5μm resolution. Images were loaded into Feature Extraction software (v8.5; Agilent) and combined with the latest array information (design-file) with default settings for all parameters including normalization. For details see Feature Extraction® Software User Manual v8, at Agilent. This results in the signal strength of the treatment (Cy5) and control (Cy3) channels, the relationship between the two channels in terms of log ratio, the associated p-value, and information concerning various quality control fields. Images and MAGE files were uploaded to Rosetta Biosoftware Resolver system, v5.0. Detailed information on the Rosetta Resolver™ system can be found at Rosetta Biosoftware (Rosetta Biosoftware, Seattle, WA), and in several published papers [31,32].

Selection considerations: Our study design involved pooled-samples for the microarrays and must be considered as a fast screening tool to select features of interest, followed by gene ontology (GO) analysis and a series of confirmatory qPCRs on the individual animals. As previously described in detail [23], criteria to select features-of-interest were based on our extensive qPCR experience with the retinal ischemia model. Power analysis of our qPCR data shows that a >1.7 fold change in expression level is statistically significant (p<0.05; power 0.80) [27,28]. To eliminate features with a low signal intensity, we added a technical log ratio error of p<0.01, assigned by Feature Extraction® software, as second criterion. A set of 19 features were excluded because of dye bias. Furthermore, 56 features were excluded for reasons of signal saturation, and 25 features were excluded because examination by qPCR showed for these genes highly variable transcript levels caused by a transfer of small amounts of lens epithelium during retina isolation [29]. Previous work has demonstrated the validity of these selection criteria [23].

Web tools: The annotation of the genes on the array was improved using web-based annotation tools DAVID-2007 (National Institute of Allergy and Infectious Diseases) [33] and Source (Stanford University, Stanford, CA). Of the 20,280 features, 92% could be linked to a UniGene cluster ID; representing 14,127 unique UniGenes. GO analysis was used to identify overrepresented or depleted functional groups of genes and was performed with GOstat [34] and DAVID-2007 [33]. Lists of selected genes-of-interest were compared with the total set of genes present on the array. Fisher’s exact test was performed to rank GO terms in GOstat. The enrichment calculation performed in DAVID, called EASE score, is a conservative adjustment to the Fisher exact probability that weighs significance in favor of themes supported by more genes [33,35,36]. The derived p-values are a score system to organize the view, and facilitate exploration rather than a strict decision making line. A p<0.01 was considered to be of potential interest.

RESULTS

Identification of genes-of-interest: The number of selected features of interest for each experimental condition, based on the change relative to the common reference sample, composed of the unoperated and sham-operated retinas, is presented in Table 1. The complete list of genes corresponding to the features-of-interest, fold-change, assigned error, p-values, and UniGene IDs for each of the time points is presented in Appendix 1. Comparison of the changes after I/R in naive animals with those in preconditioned animals (IPC-I/R) showed considerable overlap. For example, expression of c-fos was increased 29 fold 1 h after I/R and 33 fold 1 h after IPC-I/R. The effect of the preconditioning stimulus on the c-fos transcript levels is, therefore, only a relative 1.1 fold effect. Of the 254 signatures 1 h after I/R, 38% showed a significant change in the same direction after IPC-I/R. This fraction increased with time: 52%, 62%, and 77%, in the 2, 6, and 12 h I/R groups, respectively. Agglomerative clustering confirmed the similar response to ischemia in naive and conditioned retinas (data not shown). GO term analysis identified under both conditions the involvement of transcription regulation (1, 2 h), cell death (1, 2, 6, 12 h), immune response (6, 12 h), vascular development (6, 12 h), and cell motility (6, 12 h), as main categories.

We reasoned that genes with an identical response to ischemia in naive and preconditioned animals were unlikely to be critically modulated by IPC and thus did not play a pivotal role. It was therefore decided to focus on those features with a differential change in response to IPC-I/R compared to the
response after I/R only. The use of an identical common reference sample in the Cy3 channel of all arrays enabled the generation of virtual ratios from the original real ratio experiments, thus allowing the accumulation of information from all arrays in this series. In this way, gene transcripts that showed a differential response after IPC-I/R compared to I/R were selected. The number of features-of-interest identified after the re-ratio step for each reperfusion time is given in Table 1 and the complete list is presented in Appendix 1. The compiled list of all reperfusion times includes 1506 different features, reflecting 1282 unique UniGene cluster IDs. 246 UniGene IDs were altered at two time points, 109 at three, and 55 at all four reperfusion times. For further analysis; (i) for each time point, the list of features-of-interest was analyzed by GO term analysis, and (ii) a list was compiled of features with >4.0 fold change in combination with an assigned technical error of p<0.01. The latter analysis resulted in 35 upregulated and 59 downregulated features and the list is presented in Appendix 2. These genes were functionally annotated, and PubMed was surveyed for evidence supporting a possible role in cell survival as well as cell loss [33].

Gene ontology term analysis of differentially regulated genes after ischemia reperfusion between conditioned and naïve animals: For each time point, the list of features-of-interest (converted to UniGene cluster IDs) were used as input for GO term tools to identify functions/processes of interest [33,34]. The Functional Annotation Clustering tool of DAVID was used to gain insight into what biological processes were overrepresented. The results are summarized in Table 2. An extensive version can be found in Appendix 2. The clusters tRNA aminoacylation, immune response, and apoptosis were identified at three or four of the studied time points.

### Table 2. Gene Ontology Results for Genes Displaying a Differential Change as a Result of Ischemic Preconditioning

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>Group</th>
<th>C</th>
<th>P</th>
<th>ES</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA aminoacylation</td>
<td>1 h</td>
<td>8</td>
<td>0.003</td>
<td>2.51</td>
<td>Cars, Farslb, Iars, Kars, Mars, Nars, Rarsl, Tars</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td>1 h</td>
<td>17</td>
<td>0.042</td>
<td>1.17</td>
<td>Cars, Farslb, Iars, Kars, Mars, Nars, Rarsl, Tars, Bcat1, RGD1306332, Stc7al, Hibaib, Itgb2, Ppat, Typh1, Fstl2, Ddhal</td>
</tr>
<tr>
<td>tRNA aminoacylation</td>
<td>2 h</td>
<td>7</td>
<td>0.002</td>
<td>2.75</td>
<td>Cars, Farslb, Iars, Kars, Mars, Nars, Rarsl, Tars</td>
</tr>
<tr>
<td>tRNA aminoacylation</td>
<td>6 h</td>
<td>5</td>
<td>0.005</td>
<td>2.44</td>
<td>Farslb, Iars, Kars, Mars, Nars, Rarsl, Tars, Wdr46</td>
</tr>
<tr>
<td>tRNA aminoacylation</td>
<td>12 h 6</td>
<td>6.4 10-4</td>
<td>2.42</td>
<td>Cars, Iars, Mars, Nars, Rarsl, Tars, Wdr46</td>
<td></td>
</tr>
<tr>
<td>Complement activation, classical pathway</td>
<td>1 h</td>
<td>5</td>
<td>0.009</td>
<td>1.89</td>
<td>Cxcl12, Ilib, Itgam, Itgb2, Spp1</td>
</tr>
<tr>
<td>Immune response</td>
<td>2 h</td>
<td>4</td>
<td>0.036</td>
<td>0.98</td>
<td>Cldb, Cis, C4a, Serping1</td>
</tr>
<tr>
<td>Immune response</td>
<td>6 h</td>
<td>16</td>
<td>0.029</td>
<td>1.74</td>
<td>Anxal, Cldb, C3, C4d8, C5d3, Cxcl10, Fcgfr2b, Qpso3, 116, Lyx, Mar, Sele, Serping1, Stat3, Tnfrsf1a, Vezf1</td>
</tr>
<tr>
<td>Immune response</td>
<td>12 h</td>
<td>35</td>
<td>3.3 10-8</td>
<td>7.41</td>
<td>Anxal, Arts1, Atf6ap2, Cldb, C3, C5r1, Ccl17, Ccnd3, Cd3g, Cd53, Cd97, Ccrla, Csh, Cxcl12, Eprr1, Fcgri, Fcgrrb2, Fcgrr3, Fth1, Ikba, Ilib, Itgam, Itgb2, Lap1, Lyx, Oas1, Ppp2r3, Ptprc, Sl100a8, Spp1, Stat3, Tlr2, Tnfrsf1a, Tnfrsf6, Wip1</td>
</tr>
<tr>
<td>Apoptosis/programmed cell death</td>
<td>2 h</td>
<td>32</td>
<td>0.036</td>
<td>1.24</td>
<td>Aldh1a3, Anxal, Apif5, Bcl2al, Bdnf, Bircb, Cdkn1a, Cfl1, Cu5l, Dedd, Empl, Fgfr1, Hipl, Itfg1, Itgb2, Lcn2, LOC50292, Mrl, Nrl4al, Perp, Phidal, Ppp1r13b, RGD1309729, Rho2, Ripk3, Spp1, Son, Sppl, Teks2, Tnfrsf1a</td>
</tr>
<tr>
<td>Anti-apoptosis</td>
<td>6 h</td>
<td>12</td>
<td>0.012</td>
<td>1.36</td>
<td>Anxal, Apif5, Bcl2al, Bdnf, Bircb, Cdkn1a, Cfl1, Fgfr1, Itfg1, Son, Spp1</td>
</tr>
<tr>
<td>Apoptosis/programmed cell death</td>
<td>12 h</td>
<td>22</td>
<td>0.002</td>
<td>2.53</td>
<td>Aldh1al, Anxal, Apif5, Bcl2al, Crh, Cd3g, Cu12, Cu13, Dedd, Fgfr1, Fgfr3, Fgfr3, Itgb2, Lap1, Mrl, Nrl4al, Ptpn6, RGD1309729, Sphkl, Tnfrsf1a, Tnfrsf6</td>
</tr>
<tr>
<td>Anti-apoptosis</td>
<td>12 h</td>
<td>5</td>
<td>0.043</td>
<td>1.06</td>
<td>Anxal, Apif5, Bcl2al, Sphkl, Spp1</td>
</tr>
</tbody>
</table>

Full gene names and fold-change details are given in Appendix 2. In the table headings, P presents Fisher exact p-value for each biological process, and E.S. represents enrichment score for the cluster.

### Table 1. Number of Selected Features-of-Interest for Each Experimental Condition

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>Total</th>
<th>Up</th>
<th>Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemia (I/R)</td>
<td>1 h</td>
<td>254</td>
<td>223</td>
<td>31</td>
</tr>
<tr>
<td>Ischemia</td>
<td>2 h</td>
<td>216</td>
<td>182</td>
<td>34</td>
</tr>
<tr>
<td>Ischemia</td>
<td>6 h</td>
<td>920</td>
<td>592</td>
<td>328</td>
</tr>
<tr>
<td>Ischemia</td>
<td>12 h</td>
<td>1300</td>
<td>625</td>
<td>675</td>
</tr>
<tr>
<td>IPC-Ischemia (IPC-I/R)</td>
<td>1 h</td>
<td>423</td>
<td>197</td>
<td>226</td>
</tr>
<tr>
<td>IPC-Ischemia</td>
<td>2 h</td>
<td>612</td>
<td>388</td>
<td>224</td>
</tr>
<tr>
<td>IPC-Ischemia</td>
<td>6 h</td>
<td>920</td>
<td>617</td>
<td>303</td>
</tr>
<tr>
<td>IPC-Ischemia</td>
<td>12 h</td>
<td>2007</td>
<td>873</td>
<td>1134</td>
</tr>
<tr>
<td>Ratio</td>
<td>1 h</td>
<td>760</td>
<td>93</td>
<td>667</td>
</tr>
<tr>
<td>Ratio</td>
<td>2 h</td>
<td>692</td>
<td>291</td>
<td>401</td>
</tr>
<tr>
<td>Ratio</td>
<td>6 h</td>
<td>294</td>
<td>146</td>
<td>148</td>
</tr>
<tr>
<td>Ratio</td>
<td>12 h</td>
<td>382</td>
<td>174</td>
<td>208</td>
</tr>
</tbody>
</table>

A feature is defined of interest when: (i) having a dye log ratio corresponding to a difference of $>1.7$ fold change (up or down) compared to the common reference sample, and (ii) when the assigned technical error is p<0.01. The common reference sample is composed of the unoperated and sham-operated retinas. Ratio refers to the changes in the ischemic preconditioning-ischemia/reperfusion group (IPC-I/R) relative to the ischemia/reperfusion-only (I/R) group. This comparison selects for genes differentially regulated by the IPC treatment.
the alterations for these genes did not meet the selection criteria, nearly all showed a similar trend of a relatively decreased expression of IPC-I/R versus I/R (Yars, Gars, Wars, Sars1, Qrs11, Quars, Vars21, Hars2, and Rars). For Vars2, Farsla, and Dars no such trend was found. In summary, 18 out of the 21 different ARSs genes studied by microarray showed decreased transcript levels after IPC-I/R relative to I/R. The average decrease was \(-36\pm5\%\) at 1 h, \(-20\pm4\%\) at 6 h, and \(-29\pm4\%\) at 12 h (mean\(\pm\)SEM; n=18).

Transcript levels of Cars, Lars, Nar5, Wars, Tars, and Yars were also assessed by qPCR. The results are presented in Table 3. After I/R, levels gradually increased on average by 7\% at 1 h, 15\% at 2 h, and 82\% (p<0.031; Wilcoxon Matched-Pairs Signed-Ranks test; n=6) at 6 h, and by 109\% (p<0.031) at 12 h. After IPC-I/R, levels were first increased by 34\% (p<0.031) at 1 h, but thereafter, no significant changes were found. Presenting the qPCR results as re-ratio values (IPC-I/R relative to I/R) at 1 h, \(+29\pm12\%\) (p<0.05) at 2 h, \(-26\pm15\%\) at 2 h (p<0.04), \(-18\pm7\%\) at 6 h (p<0.03), and \(-37\pm3\%\) at 12 h (p<0.01).

The GO term amino acid and derivative metabolism included, in addition to ARS genes, 13 genes that paralleled the changes described for the ARSs (see Appendix 2). One of these genes was \(Slc7a1\) (solute carrier family 7 member A1), a cationic amino acid transporter. After induction of IPC, changes were found in the expression of amino acid transporter genes \(Slc7a1\), \(Slc3a2\), \(Slc6a6\), and \(Slc38a2\) [23]. In the present study these four genes showed decreased transcript levels after IPC-I/R relative to I/R at all time points of around -30\%. Two genes with a function in tRNA metabolism, \(Rg9mtd1\) and \(Trnt1\), were also relatively decreased at all time points.

**Immune response:** An overrepresentation of genes engaged in the response of the immune system was disclosed at all stages but most pronounced at 6 and 12 h (Table 2 and Appendix 2). Genes implicated in immune response were overrepresented at 6 h (16 genes) and at 12 h (35 genes). In total, 44 genes were linked to immune response of which the majority (n=36) was upregulated after IPC-I/R relative to I/R at one or more time points. The average increase was \(+64\pm18\%\) at 1 h, \(+109\pm22\%\) at 2 h, \(+78\pm9\%\) at 6 h, and \(+119\pm26\%\) at 12 h (mean\(\pm\)SEM; n=36). The average decrease observed for the other eight genes was between -20\% and -42\%. From the list of features with the highest fold-change, two more genes with a link to immune response were identified, both with increased expression: \(Serpina3n\) (associated with inflammation) and \(Lcp1\) (lymphocyte cytotoxic protein 1).

**Apoptosis:** A group of 40 different genes related to apoptosis or programmed cell death were differentially expressed at 2, 6, and 12 h (Table 2 and Appendix 2). A subgroup of 12 genes was linked to the GO term anti-apoptosis. Of the 40 genes, 25 were upregulated and 15 were downregulated. From the list of features with the highest ranking fold-change three more genes with a link to apoptosis were identified: \(Napr1\) (nuclear protein 1; up to 6.5 fold increase), \(Muc4\) (mucin 4; up to 4.8 fold increase), and \(Ppp3ca\) (protein phosphatase 3, catalytic subunit, alpha isofrom; up to 4.3 fold decrease).

**Qualitative polymerase chain reaction validation:** To validate the outcome of our array analysis, we performed qPCR assays on cDNAs of the individual retinas underlying the pooled samples used for microarray analysis. For a series of transcripts with a previously characterized alteration after I/R [27,28] together with a selection of genes from Table 2, the mean qPCR-derived change with respect to the controls was calculated as \(\log_{10}\) experimental/control. These values were

### Table 3. Changes in transcript levels of six members of the aminoacyl-tRNA synthetases family of genes as determined by real time quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>I/R</th>
<th>Transcript levels</th>
<th>Change compared to control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Lars</td>
<td>110\±9</td>
<td>94\±32</td>
</tr>
<tr>
<td>Wars</td>
<td>53\±4</td>
<td>49\±16</td>
</tr>
<tr>
<td>Tars</td>
<td>39\±3</td>
<td>44\±10</td>
</tr>
<tr>
<td>Yars</td>
<td>9\±3</td>
<td>9\±3</td>
</tr>
<tr>
<td>Cars</td>
<td>88\±10</td>
<td>99\±16</td>
</tr>
<tr>
<td>Nars</td>
<td>246\±23</td>
<td>325\±86</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IPC-I/R</th>
<th>Transcript levels</th>
<th>Change compared to control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Lars</td>
<td>86\±12</td>
<td>106\±26</td>
</tr>
<tr>
<td>Wars</td>
<td>52\±7</td>
<td>76\±16</td>
</tr>
<tr>
<td>Tars</td>
<td>24\±4</td>
<td>40\±4</td>
</tr>
<tr>
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<td>6\±1</td>
<td>8\±1</td>
</tr>
<tr>
<td>Cars</td>
<td>108\±17</td>
<td>138\±29</td>
</tr>
<tr>
<td>Nars</td>
<td>227\±29</td>
<td>241\±45</td>
</tr>
</tbody>
</table>

Asterisk indicates the outcome of the Wilcoxon matched-pairs signed-ranks test (n=6) showing a statistical significant difference p<0.031.
plotted against the corresponding log_{10} values derived from the microarrays. For the eight different experimental groups, the correlation coefficient R² ranged between 0.74 and 0.83. For all data points combined (n=291), R²=0.75. The result of the linear regression is presented in Figure 1 and the outcome of the qPCR assays is presented in Appendix 3. Of the 291 data points studied, 95 represented features-of-interest selected on the microarray results. Of these 95 data points, 72 showed a statistically significant change in the same direction based on qPCR (76%; p<0.05; student’s t-test). Of the 291 data points that were studied by both techniques, 57 data points had a statistically significant alteration based on qPCR but were not selected according to the microarray selection criteria (β=0.19). These results are in line with the outcome of our previous studies showing a good correspondence between microarray and qPCR results [23,29].

**DISCUSSION**

Retinal ischemic preconditioning offers protection against a subsequent, otherwise damaging, ischemic insult [10,11,17,19,22,38,39]. Although IPC is also observed in brain, spinal cord, and other tissues [12-15], the protective effect of IPC is particularly robust in the retina, offering full protection against functional impairment and cell loss, while cell loss is only partially prevented in other tissues. We conducted a series of microarray experiments to acquire an inventory of the changes in gene expression profile, intending to find new leads for the molecular pathways underlying the preconditioned state, previously reported [23], and to identify pathways that were modulated by preconditioning when a retina was subjected to a full ischemic insult, reported here. We hypothesized that differentially expressed genes were relevant for the effectuation of IPC-induced neuroprotection. In both unconditioned and conditioned retinas, ischemia led to changes in an increasing number of genes involving 1-2% of the features on the array at 1 h, up to 6-10% at 12 h. In contrast, the number of differentially expressed genes showed an opposite development with the highest number of genes at 1 and 2 h. Surprisingly, the extensive pattern of changes at 6 and 12 h was similar between naïve and preconditioned retinas. This showed that many changes in gene expression had no specific relationship to the occurrence of cell loss that starts to build up after 4 h of reperfusion and peaks around 6 to 12 h after ischemia [26]. However, the altered gene expression following ischemia in preconditioned retinas suggested that some, yet to be recognized, changes may occur despite the prevention of cell loss. For instance, GO term analysis showed a significant overrepresentation of genes implicated in vascular development or angiogenesis 6 and 12 h after I/R as well as IPC-I/R (e.g. Tnfa, Icam1, Il1b, and Cox2), suggesting that neovascularization may be promoted, a possible effect not addressed by morphological studies thus far [40-42].

We hypothesized that the group of differentially expressed genes were important for the protective effects induced by IPC. GO term analysis revealed an overrepresentation of genes involved in tRNA aminoacylation, immune response, and programmed cell death. GO term based selection and clustering of genes presumed that changes in a biological function were associated with concerted changes in transcript levels of the associated genes. The disadvantage of this approach was that...
genes encoding for key regulatory factors, annotated in functional context with only a limited number of other genes, did not emerge in our analysis as pathways of potential interest. For example, the observed relative decrease of Azin1 (ornithine decarboxylase antizyme inhibitor) at all time points by 1.9-4.5 fold may be of interest as Azin1 prevents the degradation of ornithine decarboxylase; its decrease may lead to a decreased production of harmful polyamines [43]. Yet Azin1 was not identified within the GO term analysis.

**tRNA aminocytlation:** The expression of ARS encoding genes was upregulated after ischemia but decreased after ischemia in preconditioned animals. At all reperfusion times a differential change was found for 18 of the 21 different ARS transcripts studied with a relative decrease of 25-35% of IPC-I/R compared to I/R. Examining the expression of several ARSs by qPCR revealed a significant increase after I/R compared to control levels, which is in line with the microarray findings. However, after IPC-I/R, the levels were not significantly different from control whereas the microarray showed decreased expression. Nevertheless, when comparing the qPCR determined ARS expression levels after IPC-I/R directly to those after I/R, a decrease of 20-35% is evident. This decrease is statistically significant at 2, 6, and 12 h reperfusion.

Previously, we showed that ARS mRNA levels decrease by 30-50% at 24-48 h after IPC induction [23]. Combined with the results of the present study we conclude that in naive animals I/R leads to increased ARS gene expression. Preconditioned retinas have lower ARS mRNA levels when subjected to I/R, levels remain decreased or only increase to levels comparable to those found in controls. The inference of these findings is that protein synthesis is necessary for the effectuation of ischemia-induced neurodegeneration and that IPC may avoid this [44-46]. Several studies have shown that the application of cycloheximide, a protein synthesis inhibitor, during the ischemic event or during subsequent reperfusion results in reduced apoptosis in brain [47-51]. Our findings suggest that suppression of protein synthesis by decreased ARSs levels may be an effective mechanism to prevent apoptosis-mediated cell loss [52].

**Immune response:** Proliferation of resident immunocompetent microglia in the retina and infiltration of leukocytes from the bloodstream are triggered by injury or inflammation and may lead to proapoptotic events [53,54]. One day after ischemia the number of cells expressing microglia/macrophage-specific markers was increased [55]. In line with their findings, we found no clear upregulation of these markers (OX42, ED1, and OX6) at 1, 2, 6, or 12 h post-ischemia. This shows that proliferation of microglia takes place after 12 h reperfusion. We were not able to conclude whether this process of proliferation was modulated by IPC.

The expression of several chemokines was shown to peak at 6 h reperfusion, which may be involved in the attraction and infiltration of leukocytes into the inner retina after ischemia at 12 h reperfusion [56]. Our microarray results confirmed increased mRNA levels at 2 h and thereafter of Ccl2 (monocyte chemoattractant protein-1), Ccl3 (macrophage inflammatory protein-1a), and Cxcl10 (interferon inducible protein-10). The finding of reduced adhesion of leukocytes after IPC suggested that inhibition of immune cell infiltration could contribute to the prevention of leukocyte-mediated damage [19,57,58]. However, our microarray and qPCR results revealed that markers for immune cell activation are upregulated after I/R and after IPC-I/R, with similar fold-change and similar time course. In addition to the aforementioned chemokines, increased expression was found for Ccl5, Ccl12, Ccl20, and Cxcl12. Other immune cell components that increased were: Vegfa [59]; Icam1, an inflammatory marker that can be induced by VEGF [40,60]; Itgb2 (CD11b), which together with Icam1 is essential for leukocyte adhesion in the retina [60]; Cox2 (Ptgs2), a proinflammatory mediator [40,61]; Ccl17, monocyte chemotactic protein 3 precursor [62]; Tyrobp, killer cell activating receptor associated protein, and Dap-12 [63]. These results show that ischemia leads to an activation of the immune system components involved in the attraction and adhesion of leukocytes in both naive and in preconditioned animals. However, the GO term immune response was still overrepresented at all time points in the group of differentially expressed genes, showing that the immune response to ischemia was modulated by prior IPC. Most differentially expressed genes were found to be upregulated in the preconditioned retinas compared to the naive retinas. Cl1qb, C1s, C3, C4a, C5r1, and Sering are implicated in complement activation along classical and alternative pathways. Cxcl2, Egr1, Il1b, Il6, and Tlr4 are associated with pro-inflammatory roles. Sele plays a role in leukocyte adhesion, and Ptprc is a leukocyte surface glycoprotein. S100a8 is an abundant protein with pro-apoptotic activity in the cytosolic fraction of neutrophils and its release is a potential biomarker for inflammation [64,65]. S100a8 was upregulated under both conditions but stronger after IPC. Only a few genes within this cluster may be related to a potential neuroprotective role effectuated by a diminished immune response. Expression of Tnfrsf1a was increased at all four time points. Released Tnfrsf1a reduced the proapoptotic effect of TNFα [66]. The observed downregulation of Tnfrsf6 prevented activation of the apoptosis effector caspase-8 [67]. The twofold upregulation at 1, 2, and 6 h of Sering1, a serine protease inhibitor also known as C1 inhibiting factor, suppressed the activity of C1s. Transcript levels of another serine protease inhibitor, Serpin3n, were upregulated by two-seven fold. This inhibitor may have a role in preventing degenerative proteolysis, which is induced by inflammatory stimuli [68].

In summary, although an activation of the immune response takes place after ischemia regardless of preceding IPC, the pattern is significantly different. Whether these differences underlie the reduced adhesion of leukocytes after IPC thus preventing infiltration and subsequent leukocyte-mediated damage, remains to be investigated [19,57,58].

**Apoptosis:** A cluster of 40 genes associated with programmed cell death were identified as differentially expressed. Functional annotation revealed several genes that were modulated as a result of IPC and may be involved in the inhibition of apoptosis-mediated cell death after ischemia. To our knowledge, at least the changes observed for the following 10 genes...
were in line with a suppressed apoptosis. The upregulation of *Anxa1* (inhibitor of phospholipase A<sub>2</sub> activity, expressed by neutrophils and monocytes and protective in myocardial I/R [69]), *Bdnf* (a cell survival promoter), *Igf1* (attenuates caspase activation and has neuroprotective effects in brain [70]), and *Muc4* (a repressor of apoptosis [71]) are all linked to anti-apoptotic effects. The downregulation *Bok1* (a pro-apoptotic Bel-2 family member [72]), *Dedd* (directs procaspases to targets [73]), *Cidec* (apoptosis inducing activity [74]) and of *Tnfrsf6* (prevents activation of the apoptosis effector caspase-8) are also in line with a suppression of apoptosis by IPC. Also, noteworthy was the 1.7 to 4.3 fold decreased expression of *Ppp3ca*, the catalytic subunit of calcineurin. Increased intracellular pressure leads to the initiation of a calcineurin-mediated mitochondrial apoptotic retinal ganglion cell death pathway in glaucoma and blockers of calcineurin are neuroprotective [75]. However, not all changes were in line with suppressed apoptosis. The decreased expression of *Birc1b*, *Perp*, *Phlda1*, and *Ripk3* might have proapoptotic consequences.

In conclusion, our microarray study on the changes in gene expression profile after preconditioning and after ischemia identified many interesting new leads for genes involved in pathways leading to either neurodegeneration or neuroprotection. One of the more intriguing findings is the pattern of changes in ARS encoding genes. The tentative neuroprotective effect of limiting translation in retinal cell survival and cell loss merits further research on the therapeutic potential in the prevention of retinal degeneration in glaucoma, diabetes, and central retinal artery occlusion.

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**REFERENCES**


