The remote ischemic preconditioning stimulus modifies inflammatory gene expression in humans

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Konstantinov, Igor E., Sara Arab, Rajesh K. Kharbanda, Jia Li, Michael M. H. Cheung, Vera Cherepanov, Gregory P. Downey, Peter P. Liu, Eva Cukerman, John G. Coles, and Andrew N. Redington. The remote ischemic preconditioning stimulus modifies inflammatory gene expression in humans. Physiol Genomics 19: 143–150, 2004. First published August 10, 2004; doi:10.1152/physiolgenomics.00046.2004.—Remote ischemic preconditioning (IPC) reduces tissue injury caused by ischemia-reperfusion (IR) in distant organs. We tested the hypothesis that remote IPC (rIPC) modifies inflammatory gene transcription in humans. Using a microarray method, we demonstrated that a simple model of brief forearm ischemia suppresses proinflammatory gene expression in circulating leukocytes. Genes encoding key proteins involved in cytokine synthesis, leukocyte chemotaxis, adhesion and migration, exocytosis, innate immunity signaling pathways, and apoptosis were all suppressed within 15 min (early phase IPC) and more so after 24 h (second window IPC). Changes in leukocyte CD11b expression measured by flow cytometry mirrored this pattern, with there being a significant (P = 0.01) reduction at 24 h. The results of this study show that the rIPC stimulus modifies leukocyte inflammatory gene expression. This effect may contribute to the protective effect of IPC against IR injury and may have broader implications in other inflammatory processes. This is the first study of human gene expression following rIPC stimulus. rIPC stimulus suppressed proinflammatory gene transcription in human leukocytes.

genes; inflammation; ischemia; leukocytes; reperfusion

ISCHEMIA–REPERFUSION (IR) injury is a ubiquitous insult in the broad spectrum of cardiovascular disease. Although cerebrovascular ischemia and myocardial infarction resulting from arterial occlusion are characterized by metabolic dysfunction, apoptosis, and necrosis, there often exists an equally destructive reperfusion injury that involves local inflammatory processes requiring leukocyte trafficking, stimulation, and exocytosis of proinflammatory chemokines. However, anti-leukocyte strategies have proven disappointing in the reduction of clinically important IR injury, possibly reflecting multiple signaling pathways involved in this process.

Ischemic preconditioning (IPC) is the most powerful innate mechanism to protect against IR injury. Observed across many mammalian species, including humans, IPC involves a brief period of sublethal local tissue ischemia that confers protection against a subsequent lethal ischemia. The early phase of IPC, referred to as “classic” preconditioning, is observed immediately after brief ischemia and is sustained for ~3 h. A delayed phase ("second window") of preconditioning has been demonstrated at 18–24 h following brief ischemia (25).

A more intriguing form of IPC with potentially greater clinical significance is “remote” IPC (rIPC). Transient tissue ischemia at a distance may confer subsequent protection of organ subjected to potentially lethal ischemia. The magnitude of its protection approaches that of local IPC, certainly in the context of myocardial infarction. Indeed, our own data demonstrated a 50% reduction in myocardial infarction in a porcine model of rIPC (17).

Given the central role of leukocytes in the pathogenesis of IR injury and the resulting systemic inflammatory response syndrome (SIRS), it would be surprising if modulation of their activity was not integral to the preconditioning effect. However, relatively little is known about the influence of preconditioning stimuli on regulation of leukocyte function. In one experimental study remote preconditioning by hindlimb ischemia reduced lung neutrophil sequestration in response to a systemic inflammatory syndrome (13). Furthermore, we have recently demonstrated that transient forearm ischemia affords protection against endothelial IR injury and suppresses circulating neutrophil activity in humans (18).

Although there is substantial evidence to suggest that the mechanisms of IPC converge on the mitochondrial ATP-sensitive potassium (K-ATP) channel, it is becoming apparent that IPC also involves changes in gene expression (8). However, there are currently no data regarding potential genomic mechanisms in rIPC. In this study we examined the effects of the stimulus of human forearm ischemia that we have used to induce local and rIPC in humans on early and late gene regulation and transcription in leukocytes.

MATERIALS AND METHODS

Subjects and Blood Samples

The study was performed on four healthy adult volunteers (3 males, 1 female), who were taking no medication (mean age, 37 yr). The rIPC protocol was as previously described (18). Briefly, the forearm was made ischemic by inflating a blood pressure cuff to 200 mmHg for three 5-min periods, separated by 5 min of reperfusion. Venous blood was drawn from the contralateral arm prior to ischemic stimulus, 15 min after completion, and 24 h later. Thus 12 samples were collected in standard sterile tripotassium-EDTA tubes (Vacutainer;
Preanalytical Solutions, Franklin Lakes, NJ) and transported on ice for immediate RNA isolation. The experimental design was approved by the Hospital for Sick Children Research Ethics Board (file number 1000000626 of November 11, 2002). The experimental design, gene lists, hierarchical trees, microarray hybridization, and statistical analysis were performed in compliance with the “minimum information about a microarray experiment” (MIAME) guidelines (3).

RNA Isolation

Total RNA (TRNA) was isolated from 12 blood samples utilizing TRIzol Reagent (GIBCO-BRL) following the manufacturer’s protocol. The quality of TRNA was assessed by Agilent 2100 Bioanalyzer (version A.02.01S1232, Agilent Technologies). Only RNA with the OD ratio of 1.99–2.0 at 260/280 was used.

Affymetrix GeneChip Hybridization and Staining

A total of 13 hybridizations were performed on the human HG-U133A GeneChip Set (Affymetrix, Santa Clara, CA) with the 12 TRNA’s from blood samples of 4 individuals at 3 different time points (control, 15 min after rIPC, 24 h after rIPC) and 1 reference TRNA (Stratagene) as a universal control. Samples were prepared for hybridization according to standard Affymetrix instructions and performed at the genomic core center at the Hospital for Sick Children.

Briefly, a primer encoding the T7 RNA polymerase promoter linked to oligo-dT7 was used to prime double-stranded cDNA synthesis from each mRNA sample using SuperScript II RNase H− reverse transcriptase (Life Technologies, Rockville, MD). Each purified (Qiaquick kit, Qiagen) double-stranded cDNA was in vitro transcribed using T7 RNA polymerase (T7 kit; Enzo Biochemicals, New York, NY), incorporating biotin-UTP (Enzo) into the cRNAs, followed by purification using RNeasy (Qiagen) and quantitated by measuring absorption at 260 nm/280 nm. Samples were fragmented and hybridized to the GeneChip for 16 h at 45°C and scanned (GeneArray scanner, Affymetrix). MicroArray Suite version 5.0 (MAS 5.0; Affymetrix) was used to scale intensities across the GeneChips to 150 fluorescence units and to determine expression values for each gene on the GeneChip. The expression value for each gene was determined by calculating the average of differences (perfect match intensity minus mismatch intensity) of the probe pairs in use for the gene.

Affymetrix GeneChip Data Analysis

Gene analysis software. Data analysis was performed using two independent software packages, GeneChip (Affymetrix) and GeneSpring (Silicon Genetics, Redwood, CA).

Data analysis. Scanned raw data were processed with Affymetrix MAS 5.0 software. The average intensity value for each probe set, which directly correlates with mRNA abundance, was calculated as an average of fluorescence differences for each perfectly matched vs. single-nucleotide mismatched probe. To test the integrity of the starting RNA, we examined the signal intensity ratio for the 3′ probe set over the 5′ probe set for the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For the 13 arrays used in this study, the 3′-to-5′ ratios were 1.3 ± 0.07 and 0.97 ± 0.06 for β-actin and GAPDH, respectively. Once sample quality was demonstrated, those genes with consistently present calls were considered. To monitor the expression of chosen genes over the different experimental time points, data obtained from MAS 5.0 absolute analyses of all the individual arrays were analyzed and clustered using GeneSpring (http://www.silicongenetics.com).

GeneSpring 6.1 was then used for normalization, each sample was normalized to its control (untreated), and then genes were filtered first based on P < 0.05; then one-way ANOVA (not equal variance) was performed on these genes, and, finally, 1.5-fold up- or downregulated genes in 24 h vs. control generated 169 genes that were used for hierarchical clustering (Fig. 1). Each sample was analyzed individually, and none of the methods was applied to pooled data.

Microarray Validation

Validation of the microarray method was performed using reverse transcription polymerase chain reaction (RT-PCR). It was performed on TRNA isolated from blood in each of four individuals for one of the upregulated genes, calpastatin. The experiments were performed in duplicates. PCR results for calpastatin expression demonstrated the trend identical to microarray results. Expression profile of calpastatin in all samples were confirmed using one-step RT-PCR (Qiagen). Primer sequences were designed using GeneRunner program directed toward the 3′ sequence of the gene. The primers sequences for calpastatin was as follows: forward, TTAGTTTCTCTGTAGGGCTGCT; reverse, GTGAACTGCGCTTCCAACCGG. The GAPDH was used as an internal control for every PT-PCR for each individual sample.

Assessment of Neutrophil Activation

Neutrophil activation was assessed by the level of expression of CD11b (α-chain of the integrin adhesion molecule CD11b/CD18, Mac-1) and measured by fluorescence intensity of FITC-conjugated IgG1 monoclonal antibody directed against CD11b (Serotec), expressed as the median fluorescence intensity (MFI) of the total leukocyte population. Samples were analyzed by flow cytometry within 1 h of collection as previously described (18).

RESULTS

Effects on Gene Expression

RNA isolated from blood was analyzed on oligonucleotide arrays representing over 50% of the estimated total number of human genes (Affymetrix, http://www.affymetrix.com). Data filtering (P < 0.05) and hierarchical clustering (using an average linkage algorithm: GeneSpring software, Silicon Genetics) identified significant changes in the expression of ~169 genes over the course of the study period (Fig. 1).
The expression of some genes varied among individuals, particularly at the initial stage of the response (15 min); however, the majority of differentially expressed genes depicted identical patterns at 24 h. Our complete microarray data and analysis algorithm are available on the CHFNET Group website (http://www.chfnet.ca/supplementkonst.asp).

The expression of 35 genes was upregulated at both 15 min and 24 h after forearm ischemia, while the expression of 134 genes was suppressed. The key genes involved in innate immune responses, TNF-signaling pathways, leukocyte adhesion, chemotaxis, and exocytosis are listed in Table 1. Of particular interest to the potential anti-inflammatory influence of the genomic responses observed was the upregulation of heat shock protein (HSP) 70 and calpastatin, as well as the downregulation of genes of innate immunity responses (TLR4) and TNF signaling pathway (TNFR6). Genes encoding proteins crucial to intracellular proinflammatory mechanisms, e.g., programmed cell death (caspase-8), leukocyte extravasation (PI3KCA), and secretory granule release (SNP-23) were also significantly downregulated.

**Effects on CD11b Expression**

At baseline, the MFI for CD11b expression was 17.0 ± 5.2 U. After 15 min of reperfusion CD11b expression decreased to 11.5 ± 4.7 U but did not reach statistical significance (P = 0.23). It decreased further at 24 h to 5.3 ± 0.9 U, and the difference was significant compared with baseline (P = 0.01).

**Table 1. Transcription of the key genes of inflammatory response**

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Values are mean normalized intensity, with SD in parentheses. Repeated genes are due to multiple presence sites on the Affymetrix GeneChip. The real antisense target is listed first, followed by similarity-based antisense (*).
DISCUSSION

Our data demonstrate for the first time that the rIPC stimulus achieved by transient forearm ischemia has predominantly downregulatory effects on gene expression in circulating human leukocytes. The magnitude of change associated with our in vivo human experiments approaches that reported, using identical microarray techniques, in ex vivo neutrophil (8) and eosinophil (41) preparations subjected to supraphysiological stimuli. Approximately 30 key genes known to be involved in leukocyte activation and innate immunity, cell adhesion, intracellular signaling, and effector pathways such as exocytosis were affected.

HSP70 Transcription and TLR Pathway Genes

Expression of HSP70 was upregulated at 15 min and more markedly at 24 h (Table 1 and Fig. 2). HSP70 activates NF-κB via toll-like receptor (TLR) stimulation, utilizing its toll/IL-1R/plan R gene homology (TIR) domain, myeloid differentiation protein (MyD88), interleukin (IL)-1 receptor-associated kinase (IRAK), TNF receptor-associated family (TRAF), and IκB kinases (IKK)(41) (Table 1 and Fig. 2). It is of interest that both HSP70 and bacterial lipopolysaccharide (LPS) transduce the signal through a glycosylphosphatidylinositol-anchored membrane protein, CD14 receptor (Figs. 2 and 3). CD14 associated with the cell surface by means of a glycolipid linkage and is not capable of generating a transmembrane signal. It is likely that the HSP70 or LPS attach to LPS binding protein (LBP)-CD14 complex and activate TLR4, which in turn, via its TIR domain, signals through the adapter protein MyD88 and the serine kinase, IRAK. MD-2, a secreted protein that binds to the extracellular domain of TLR4 as well as multiple leucine-rich repeats of both TLR4 and CD14, might facilitate protein-protein reaction and are important in the signaling (1, 41). Neither MyD88 or IRAK genes were suppressed in our study. HSP70 also has an important intracellular cytoprotective role against the wide variety of inflammatory stimuli (4). Our finding of suppression of the key genes involved in TNF-α synthesis (see below) is consistent with the observations that prior exposure to either LPS (43) or HSP70 (21, 45) decreases subsequent TNF-α production. TLR4 is

Fig. 2. Innate immunity pathways. Red indicates upregulated genes, yellow indicates gene expression was not changed, and green indicates downregulated genes. HSP, heat shock protein; LPS, lipopolysaccharide; LBP, LPS binding protein; TLR, toll-like receptor; IRAK, interleukin (IL)-1 receptor associated kinase; TIR, toll/IL-1/plan R homology domain; TNF, tumor necrosis factor; TRAF, TNF receptor-associated family; TRADD, TNF receptor-associated death domain; IKK, IκB kinase; and MnSOD, manganese superoxide dismutase. For further detailed definitions of abbreviations, refer to the main text and Table 1.
involved in LPS-induced oxidative burst in neutrophils and has a role in IR injury (31, 35). We observed significant down-regulation of the TLR4 gene at both 15 min and 24 h (Table 1).

TNF-α Signaling Pathway

TNF-α plays a key role in postischemic damage in various organs including the brain, heart, liver, and lung. TNF pretreatment results in reduction of IR injury and correlates with an increase in myocardial antioxidant, manganese superoxide dismutase (MnSOD) activity (29, 49). MnSOD is also induced by TNF-α, IL-1, LPS, and HSP70 (Fig. 2). TNF also plays an important role in late IPC (7, 49) by activation of the NF-κB, which in turn upregulates MnSOD synthesis (48). Although both TNF and MnSOD may be induced via the CD14 and TLR4 receptors (43), cells have a specific TNF receptor (TNF-R1) through which TNF induces NF-κB-dependent MnSOD synthesis (48). Although both TNF and MnSOD may be induced via the CD14 and TLR4 receptors (43), cells have a specific TNF receptor (TNF-R1) through which TNF induces NF-κB-dependent MnSOD synthesis (48).

The reduction in transforming growth factor-β-activated kinase 1 (TAK1) is important in IκB kinase-mediated activation of the NF-κB pathway (40). TNF-α-induced activation of the NF-κB is associated with binding of TAK1 to TRAF, and both IKKα and IKKβ and thus play a role in TNF-α signaling (Fig. 2). TAK1-binding protein (TAB2) acts as an adapter linking TAK1 to TRAF. Both TAK1 and TAB2 gene expression was suppressed (Table 1).

TNF Synthesis

All three key kinases involved in TNF synthesis, MAPKAPK2, MEKK2, and MAP3K8, were suppressed (Table 1). It
is established that the mitogen-activated protein kinase (MAPK) p38 regulates cytokines, including TNF-α production at the translational level, and the effect of MAPK p38 is mediated by mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) (19). Indeed, elimination of MAPKAPK2 in a mouse knockout model results in significantly decreased MAPK p38 levels in most tissues, but especially in heart and liver, and reduced synthesis of TNF-α protein (20). TNF production was restored by adding catalytically active MAPKAPK2, but not MAPK p38 alone (20). In addition, MAP/ERK kinase (MEK) kinase (MEKK) 2 activation is critical for c-Jun-N-terminal kinase (JNK) activation and TNF-α production (5).

Apoptosis

IR injury initiates caspase-8-mediated apoptosis (30). Caspase-8 induction is mediated by TNF via TNF-R1 and Fas-associated death domain (FADD) (Fig. 2). Activated neutrophils and macrophages stimulate Fas via production of reactive oxygen species (30). In our study there was a marked reduction of caspase-8 and caspase-8-associated protein 2 (CASP8AP2) gene expression within 15 min of transient forearm ischemia, which persisted at 24 h (Table 1). MASPAP2 plays a regulatory role in Fas-mediated apoptosis (6).

Exocytosis

Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) are core machinery for membrane fusion during exocytosis (Fig. 2) in many cells, including mast cells and human neutrophils (11, 28). A synaptosome-associated protein (SNAP-23) is a target SNARE (t-SNARE) with crucial involvement in mast cell and neutrophil exocytosis (11, 26, 28). We observed a threefold reduction in SNAP-23 gene expression at 24 h following forearm ischemia (Table 1). Expression of the genes encoding other exocytosis-related SNAREs [syntaxin 3, vesicle-associated membrane protein 3 (VAMP3 or cellubrevin)] and coated vesicle membrane protein (P24A) was also significantly suppressed (P < 0.005) (Table 1). Relocation of SNAP-23 from cell surface to the secretory granules membrane is prerequisite for exocytosis (11). As secretory granules in unstimulated mast cells lack SNAP-23, formation of ternary SNARE complexes with syntaxin 3 and VAMP relative is not possible until SNAP-23 relocates (Fig. 2). This implies that relocation of SNAP-23 controls entry of mast cell granules into the “fusion-ready” state (11).

Expression of CD11b

This study was designed primarily to examine potential changes in gene expression imposed by this stimulus. The functional implications of these genetic changes need to be examined systematically. However, CD11b receptor is expressed on the surface of activated leukocytes by exocytosis (Fig. 2). We have previously shown, in a model of local preconditioning, that a similar IPC stimulus reduces early CD11b expression (18). Chemokines attract circulating leukocytes to the microvasculature by triggering inside-out signal transduction pathways leading to integrin-dependent adhesion (43) (Fig. 3). Integrin activation by chemokines is very rapid and allows binding of rolling cells to the endothelium within minutes (23, 43). Chemokine activity may be mediated through activation of the G protein-coupled receptor TM7LN4, the expression of which was reduced in our study (15) (Table 1). Similarly, there was a significant suppression of gene expression of lymphocyte cytosolic protein 2 (SLP-76), an important adaptor of T-cell receptor (TCR) that plays a central role in normal T-cell and mast cells activation (44). It is of interest that α4-integrin (Fig. 3) functions not only as an adhesion molecule, but also mediates neutrophil free radical injury to cardiac myocytes (32, 33). Addition of an anti-α4-integrin antibody completely inhibited oxidant production, even though neutrophils continue to adhere to the myocyte via β2-integrins (CD18) (32). Neutrophil migration across mouse cardiac endothelium persists in the absence of CD18 but is completely blocked by antibodies against α4-integrin or VCAM-1 (2). We showed suppression of the gene for α4-integrin (CD49D) by twofold in 24 h (Table 1). Chemokine CC receptor 2 (CKR2a) plays a central role in neutrophil and macrophage migration (22, 27) and respiratory burst in human neutrophils (33). Profound reduction in leucocyte adhesion with severely decreased monocyte and neutrophil extravasation was observed in mice deficient in CKR2a (22, 27), which was also suppressed by forearm ischemia in our study (Table 1 and Fig. 3). The phosphoinositide 3-kinase (P13K) catalytic subunit p110 (P13KCA)-δ is expressed in neutrophils and facilitates their accumulation at sites of inflammation by contributing to chemoattractant-directed migration. The blockade or gene deletion of P13KCA reduces neutrophil influx into tissues by diminishing their attachment to and migration across endothelium (34). We observed significant suppression of P13KCA gene expression (Table 1).

Calpain regulates β1 (α4)-integrin-mediated leucocyte adhesion (9, 36). Leukocyte α4-integrin, and its endothelial ligand vascular cell adhesion molecule (VCAM)-1, are crucial in neutrophil migration across endothelium (2). Selective inhibition of calpain attenuates neutrophil-mediated myocardial IR injury (14). We observed late upregulation of the endogenous calpain inhibitor, calpastatin (CAST), which was only significant at 24 h (Table 1). Gene expression of platelet endothelial cell adhesion molecule (PECAM1, CD31) was suppressed (Table 1). We have recently observed a significant reduction in blood level of troponin I (TnI) in animal model by rIPC after cardiopulmonary bypass (CPB) (16). Because calpain degrades TnI, activation of calpastatin gene expression is consistent with preservation of TnI by rIPC (16) or glucocorticoids (38). PECAM1 is expressed on endothelial cells, leukocytes, and platelets. Blockade of PECAM1 reduces IR injury via inhibition of neutrophil migration (12). Finally, gene expression of ADAM 8 and 10, both leukocyte adhesion molecules, and MAPKAPK2, inhibition of which reduces filopodium formation and migration of macrophages (20), was suppressed (Table 1).
Tissue Inhibitor of Matrix Metalloprotease (TIMP)

TIMP-1 inhibits matrix metalloproteinases (MMP) 2 and 9, both of which play a crucial role in acute myocardial dysfunction after IR injury (37, 46). TIMP-1 is also known to inhibit proteases of the ADAM (“a disintegrin and metalloprotease”) family, including ADAM 10. TIMP gene expression was upregulated in the present study, whereas ADAM 8 and 10 genes were suppressed (Table 1).

Although the proteomic and functional implications of these findings are yet to be fully elaborated, it is clear from many other studies that leukocytes play a central role in IR injury. In our experiments, the induction of neutrophil CD11b expression and migration in these models. By implication, it is intriguing to speculate that modification of white cell responses using this stimulus may have more diverse applications in human disease.

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