Cardiopulmonary Bypass Decreases G Protein–Coupled Receptor Kinase Activity and Expression in Human Peripheral Blood Mononuclear Cells

Scott A. Hagen, M.D.,* Amy L. Kondyra, B.A.,† Hilary P. Grocott, M.D.,‡ Habib El-Moalem, Ph.D.,† Daniel Bainbridge, M.D.,§ Joseph P. Mathew, M.D.,‡ Mark F. Newman, M.D.,¶ Joseph G. Reves, M.D.,** Debra A. Schwinn, M.D.,∥ Madan M. Kwatra, Ph.D.‡

** Background:** Cardiopulmonary bypass (CPB) has been implicated in the development of organ injury associated with cardiac surgery. At the molecular level, CPB is accompanied by a pronounced proinflammatory response including an increase in plasma interleukin (IL)-6. The IL-6 has been shown to be increased in rheumatoid arthritis, a chronic inflammatory disease, where it has been implicated in decreasing G protein–coupled receptor kinases (GRKs) in peripheral blood mononuclear cells. Since IL-6 is substantially increased after CPB, the study tested whether the increase of IL-6 during CPB leads to a decrease of GRKs in mononuclear cells. This is important because GRKs regulate the function of G protein–coupled receptors involved in inflammation.

**Methods:** Fifteen patients had blood withdrawn before CPB, 2 h after CPB, and on postoperative day one (POD1). Plasma IL-6 concentrations were determined by enzyme-linked immunosorbent assay. The GRK protein expression and activity were determined by Western blot and phosphorylation of rhodopsin using [γ-32P] adenosine triphosphate, respectively.

**Results:** Plasma IL-6 increased over 20-fold after CPB and remained increased on POD1. Cytosolic GRK activity in mononuclear cells decreased by 39 ± 29% cytosolic GRK2 and membrane-bound GRK6 decreased by 90 ± 15 and 65 ± 43%, respectively. The GRK activity and expression of GRK2/GRK6 on POD1 returned to basal levels in many but not all patients.

**Conclusions:** The CPB causes a profound decrease in mononuclear cell GRKs, and the recovery of these kinases on POD1 is quite variable. The significance of the variable recovery of GRKs after CPB and their potential role as a marker of clinical outcome deserves further investigation.

While cardiopulmonary bypass (CPB) has made it possible to perform a variety of surgeries on the human heart, it has been implicated in a number of postoperative complications including atrial fibrillation, cognitive impairment, and other organ dysfunction.1–4 To understand the molecular basis of these postoperative complications, several investigators have examined biochemical changes in blood in the perioperative period. One prominent change associated with CPB is a steep increase in plasma proinflammatory cytokines, including interleukin (IL)-6.5–6 These inflammatory cytokines mediate a systemic inflammatory response that can result in multiorgan dysfunction. Increases in IL-6 following CPB have been associated with circulatory dysregulation, myocardial dysfunction and ischemia.6–9 Increases in IL-6 concentrations are also associated with aging and outcomes from the systemic inflammatory response syndrome, sepsis, and adult respiratory distress syndrome.10–13

The IL-6 can contribute to cellular injury by several mechanisms, including upregulation of adhesion molecules on endothelium and neutrophils, as well as expression of inducible nitric oxide.14–17 A recent study links increased plasma IL-6 with a dysfunction in the regulation of G protein–coupled receptors (GPCRs) in peripheral blood mononuclear cells.18 This study demonstrated that patients with rheumatoid arthritis have decreased concentrations of G protein–coupled receptor kinases (GRKs), a family of six isozymes numbered GRK1 to GRK6, in their mononuclear cells. The decrease was attributed to IL-6 because mononuclear cells from healthy donors, when exposed to IL-6, showed a decrease in GRK2 expression.18 These data indicate that IL-6, which is synthesized in mononuclear cells, has mononuclear cells as one of its targets. The effect of IL-6 on GRKs is likely to be important because these kinases play a key regulatory role in the desensitization or attenuation of signaling through GPCRs, including GPCRs for substance P, IL-8, and chemokines that are known to cause inflammation.19,20 Since IL-6 is increased during CPB, we sought to determine whether GRKs in peripheral blood mononuclear cells are affected by CPB.

**Materials and Methods**

**Patient Data and Collection of Blood Samples**

After written informed consent was obtained, fifteen patients were enrolled in this study approved by the Institutional Review Board. The patients underwent surgery involving CPB and were enrolled successively without exclusions for patient condition or type of surgery. Blood samples (15 ml) were collected in EDTA-coated tubes at the following times: before surgery (PRE), 2 h after completion of CPB (POST), and 24 h after completion of CPB (POD1). Samples were transported to the laboratory at room temperature. Patient records were reviewed to gather demographic data, preoperative di-
agnosis, surgical procedure, length of CPB, length of hospital stay, and postoperative complications.

**Isolation of Plasma and Mononuclear Cells**

An 800 μl portion of each sample was centrifuged at 1500 × g to obtain plasma used for the IL-6 assay. The remaining blood was used to isolate peripheral blood mononuclear cells over Ficoll-Hypaque Histopaque® (Böyum, St. Louis, MO) density gradients, as described by Böyum. Mononuclear cells and plasma were stored at −80°C until used.

**Preparation of Cytosolic and Membrane Fractions of Mononuclear Cells**

Mononuclear cells were thawed, then suspended in 0.5–1.0 ml of ice-cold buffer A (20 mM Tris-HCl [pH 7.4], 2 mM EDTA, containing the protease inhibitors 100 μM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 150 μg/ml benzamidinede); homogenized using a hand-held tissue disruptor on low speed; and centrifuged at 4°C at 45,000g for 15 min. The supernatant, representing the cytosolic fraction, was stored at −80°C until analysis. The membrane pellet was resuspended in 0.5 ml buffer A and stored at −80°C.

**Measurement of IL-6 in Plasma**

The concentration of plasma IL-6 was determined by enzyme-linked immunosorbent assay using a commercially available kit following the manufacturer instructions (Quantikine HS, R&D Systems, Minneapolis, MN).

**Measurement of GRK Activity in Mononuclear Cell Cytosol**

The GRK activity in the cytosolic fraction was assessed using light-dependent phosphorylation of rhodopsin in urea-washed bovine rod outer segments (ROS), as described previously. Briefly, 6 μg of cytosolic protein from mononuclear cells and 5 μg of ROS proteins were suspended in a total volume of 50 μl consisting of 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 2 mM EDTA, and [γ-32P] adenosine triphosphate (final concentration 100 μM, specific activity 2–4 cpn/fmol). The reaction was allowed to proceed for 5 min at 30°C and was then stopped by adding 50 μl of sample buffer (25 mM Tris-HCl [pH 6.8], 10% glycerol, 8% sodium dodecyl sulfate, 0.0125% Bromophenol Blue). Phosphorylated rhodopsin was separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and visualized by autoradiography. The rhodopsin band was excised from the dried gels and counted by liquid scintillation spectroscopy. Background phosphorylation was determined by performing phosphorylation of rhodopsin in the absence of mononuclear cell cytosol, and 32P incorporated into rhodopsin was subtracted from 32P incorporated into rhodopsin in the presence of mononuclear cell cytosol. Results are expressed as fmol 32P incorporated into rhodopsin · minute⁻¹ · μg cytosolic protein⁻¹, and as a percentage of baseline (PRE) activity.

**Measurement of GRK2 and GRK6 Proteins in Mononuclear Cells by Immunoblotting**

The expression of GRK2 and GRK6 proteins in the cytosolic and membrane fractions of mononuclear cells was determined by Western blot. Samples containing 30 μg of protein were placed in SDS-sample buffer, incubated for 15 min at 37°C, and subjected to electrophoresis on 10% SDS-PAGE gels. The separated proteins were transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were incubated for 1 h in a 1:2000 dilution of primary rabbit polyclonal antibodies to GRK2 or GRK6 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by 1 h incubation in a 1:2000 dilution of antirabbit secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactivity was detected by enhanced chemiluminescence (Pierce, Rockford, IL), and quantified by densitometry using Quantity One software (Bio-Rad Laboratories, Hercules, CA). The signals were determined to be in the linear range for chemiluminescence. The GRK protein expression in POST and POD1 samples is reported as a percentage of the PRE sample for each individual. Purified recombinant GRK2, prepared as described previously, was used as standard in GRK2 immunoblots. Recombinant GRK6 in Sf9 membranes was used as standard in GRK6 immunoblots. To express GRK6 in Sf9 cells, human GRK6 cDNA was excised from ATCC clone HFBCB24 (ATCC, Rockville, MD) with EcoRI/Bsp 120I (Apa I) and subcloned into PV1393 digested with EcoRI/NotI. A recombinant baculovirus containing GRK6 was isolated using the protocol provided with the BaculoGold transfection kit (Pharmingen, San Diego, CA), and it was used to infect Sf9 cells. The growth of Sf9 cells and preparation of Sf9 membranes have been described previously.

Only GRK2 and GRK6 were measured because the commercially available antibody to GRK3 also reacted with purified GRK2, and levels of GRK5 were too low to quantify. Whether our GRK2 antibody cross-reacts with GRK3 could not be assessed because purified GRK3 was not available. The GRK6 was analyzed only in mononuclear cell membranes because it is a membrane-associated enzyme. The GRK1 and GRK4 were not examined because these GRKs are only expressed in retinal and testicular tissue, respectively.

**Statistical Analysis**

The GRK activity and protein expression for each subject are reported as a percentage of PRE levels and expressed as mean ± SD. An exact Wilcoxon signed rank test or paired t test was used to study change in GRKs and IL-6 values between PRE, POST and POD1.
samples; we applied the Bonferroni correction to these changes and a \( P \) value of less than 0.0167 was considered significant for these tests. The relationship between these measured variables were determined using exact tests of Kendall \( \tau_b \) correlations, simple multiple linear regression, and separate mixed models. A \( P \) value of less than 0.05 was considered significant for these tests. Data were analyzed using SAS software (SAS, Cary, NC) and STATXACT (Boston, MA) software.

**Results**

Table 1 presents patient demographic data, surgical procedure, length of CPB, length of postoperative hospital stay and postoperative complications.

**Plasma IL-6 Increases after CPB**

Plasma IL-6 increased more than 20-fold after CPB from 2.9 to 62.0 pg/mL \( (P < 0.0001) \) and remained increased to 88.6 pg/mL on POD1 \( (P < 0.0001) \) (fig. 1).

**CPB Decreases GRK Activity in Mononuclear Cells**

Figure 2A shows that rhodopsin phosphorylation, which represents GRK activity, decreased substantially in the POST sample and remains low in the POD1 sample. Average kinase activity in PRE samples was 7.5 \( \pm \) 3.5 fmol \( 32 \text{P} \) incorporated into rhodopsin \( \cdot \) minute \(^{-1} \cdot \mu\text{g cytosolic protein} \)\(^{-1} \), and it decreased by 39 \( \pm \) 29\% after CPB \( (P = 0.0014) \). The CPB-induced decrease in GRK activity was seen in all but one patient, but the recovery was quite variable among patients (fig. 2B).

**CPB Decreases GRK2 Protein**

We determined whether the observed decrease in GRK activity after CPB was due to a decrease in GRK2 protein. Figure 3 shows that cytosolic GRK2 expression decreased dramatically after CPB by 90 \( \pm \) 15\% \( (P = 0.0001) \), indicating that the decrease in cytosolic GRK activity after CPB could be attributed to a decline in GRK2 protein. The decrease in cytosolic GRK2 was not due to GRK2 translocation to membrane because membrane-associated GRK2 was also significantly decreased after CPB by 78 \( \pm \) 27\% \( (P = 0.002, \text{data not shown}) \). It should be pointed out that the decrease in GRK2 protein in POST samples (fig. 3B) was much greater than the decrease in GRK activity (fig. 2B). The disparity between measured GRK activity in the cytosol and the expression of GRK2 protein in the cytosol indicates that other kinases contribute to the phosphorylation of rhodopsin.

![Figure 1. Effect of cardiopulmonary bypass (CPB) on plasma interleukin (IL)-6 (n = 11). Plasma IL-6 was measured as described in the methods section. The increase in IL-6 from PRE to POST and POD1 samples was statistically significant (\( P < 0.0001 \)). The increase in IL-6 from POST to POD1 did not reach statistical significance (\( P = 0.035 \)). PRE = before surgery; POST = 2 h after completion of CPB; POD1 = 24 h after completion of CPB.](image)
As with GRK activity, the recovery of GRK2 on POD1 differed considerably between patients (fig. 3B).

**Effect of CPB on G proteins**

To determine whether the decrease in GRK2 after CPB was protein-specific, we examined the effect of CPB on the G proteins $G_{so}$ and $G_{io}$ (proteins which, like GRKs, are part of the GPCR signaling network).\textsuperscript{19,28} As Figure 4 shows, the effect of CPB on $G_{so}$ and $G_{io}$ proteins does not parallel the effect on GRK2. Thus the effect of CPB on GRK2 decline is GRK2 specific and is not due to a CPB-induced global degradation of proteins. Our finding that CPB had a variable effect on $G_{so}$ and $G_{io}$ proteins in mononuclear cells is consistent with a recent report by Tittelbach et al. which found that $G_{so}$ and $G_{io}$ protein expression in peripheral blood mononuclear cells was increased in some patients after CPB, and decreased in other patients.\textsuperscript{28}

**CPB Decreases GRK6**

Figure 5 shows that membrane-bound GRK6 decreases after CPB and the average decrease in GRK6 is $65 \pm 43\%$ from PRE levels ($P = 0.0015$). As with GRK2 and GRK activity, the recovery of GRK6 on POD1 varied considerably between patients (fig. 5B).

**Correlation between IL-6 and GRKs**

A negative correlation between cytosolic GRK activity and plasma IL-6 was detected when a mixed model analysis was performed ($P = 0.0017$) and a negative correlation was also found between GRK6 expression and IL-6 using simple linear regression ($P = 0.0181$). However, we did not find a statistically significant correlation between changes in GRK2 expression and plasma IL-6, presumably due to our small sample size.

Fig. 2. Effect of cardiopulmonary bypass (CPB) on G protein–coupled receptor kinase (GRK) activity in mononuclear cells ($n = 15$). (A) Autoradiogram from patient 10 showing GRK activity in cytosolic fraction of mononuclear cells as measured by phosphorylation of rhodopsin in urea-washed bovine rod outer segment (ROS). Lane 1, phosphorylation of ROS with purified recombinant GRK2; lane 2, phoshorylation of ROS in the absence of cytosolic mononuclear cell fraction; lane 3, phosphorylation of ROS with pre-CPB cytosol (PRE); lane 4, phosphorylation of ROS with post-CPB cytosol (POST); lane 5, phosphorylation of ROS with postoperative day 1 (POD1) cytosol. (B) The GRK activity (as a percent of PRE) in POST and POD1 samples from individual patients. The numbers on the right correspond to patient numbers shown in table 1.

Fig. 3. Effect of cardiopulmonary bypass (CPB) on GRK2 protein in mononuclear cell cytosol ($n = 15$). (A) GRK2 immunoreactivity in mononuclear cell cytosol from patients 7 and 4. The immunoblotting was carried out as described in the methods section. GRK2 lane, $100 \, \text{ng}$ of purified GRK2; PRE lanes, $30 \, \text{g}$ mononuclear cell cytosol protein from pre-CPB sample; POST lanes, $30 \, \text{g}$ mononuclear cell cytosol protein from post-CPB samples; POD1 lanes, $30 \, \text{g}$ mononuclear cell cytosol protein from POD1 samples. (B) GRK2 protein expression in POST and POD1 samples from individual patients. Patient numbers are shown to the right. PRE = before surgery; POST = 2 h after completion of CPB; POD1 = 24 h after completion of CPB.
Discussion

In this study, we provide the first evidence that cardiac surgery involving CPB decreases GRK activity and expression in peripheral blood mononuclear cells. More importantly, we find that the recovery of GRK activity and expression on POD1 is highly variable among patients, with some patients showing little or no recovery. This finding is interesting because a poor recovery of GRKs after CPB may slow the desensitization of GPCRs on mononuclear cells that propagate inflammation, thus contributing to postoperative morbidity. Testing this possibility would require a larger prospective study.

The main purpose of our study was to determine if the acute increase in IL-6 seen after CPB is associated with a decrease in GRKs in peripheral blood mononuclear cells. We found that the CPB-induced increase in IL-6 had a negative correlation with GRK activity and GRK6 expression. However, our data suggest that while IL-6 may contribute to the observed decrease in GRKs, these kinases recover in many patients on POD1 despite increased concentrations of plasma IL-6. This observation differs from the persistent decrease in GRK2 demonstrated by Lombardi et al. after exposure of mononuclear cells to IL-6 in vitro. It seems that under in vivo conditions, IL-6 may be the mechanism for the initial decrease in GRKs, but its effects are attenuated with time, possibly due to the release of antiinflammatory molecules such as IL-10. It should also be kept in mind that the observed effect on GRKs could be mediated through other cytokines or biomolecules since CPB is known to be accompanied with a profound inflammatory response. Clearly, further studies are needed to delineate the molecular events responsible for the observed decline in GRKs after CPB.

In conclusion, we have demonstrated that CPB is associated with a significant decrease in GRK activity as well as in GRK2 and GRK6 protein expression in mononuclear cells. The significant variability among patients in the decline and recovery of GRKs after CPB should be further investigated as potential markers for the development of postoperative complications. Finally, it should be pointed out that the decline in GRKs seen in mononuclear cells after CPB may or may not occur in other tissues.
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References


