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Adjuvant Arthritis Induces Down-Regulation of G Protein-Coupled Receptor Kinases in the Immune System

Maria Stella Lombardi,²* Annemieke Kavelaars, * Pieter M. Cobelens, * Reinhold E. Schmidt, † Manfred Schedlowski, ‡ and Cobi J. Heijnen*  

G protein-coupled receptors (GPCRs) play a crucial role in the regulation of the immune response by, e.g., chemokines, PGs, and β2-adrenergic agonists. The responsiveness of these GPCRs is turned off by the family of G protein-coupled receptor kinases (GRK1–6). These kinases act by phosphorylating the GPCR in an agonist-dependent manner, resulting in homologous desensitization of the receptor. Although GRKs are widely expressed throughout the body, leukocytes express relatively high levels of GRKs, in particular GRK2, -3, and -6. We investigated whether in vivo the inflammatory disease adjuvant arthritis (AA) induces changes in GRK expression and function in the immune system. In addition, we analyzed whether the systemic effects of AA also involve changes in GRKs in nonimmune organs. At the peak of the inflammatory process, we observed a profound down-regulation of GRK2, -3, and -6 in splenocytes and mesenteric lymph node cells from AA rats. Interestingly, no changes in GRK were observed in thymocytes and in nonimmune organs such as heart and pituitary. During the remission phase of AA, GRK levels in spleen and mesenteric lymph nodes are returning to baseline levels. The decrease in GRK2 at the peak of AA is restricted to CD45RA+ B cells and CD4+ T cells, and was not observed in CD8+ T cells. In conclusion, we demonstrate in this study, for the first time, that an inflammatory process in vivo induces a tissue-specific down-regulation of GRKs in the immune system. The Journal of Immunology, 2001, 166: 1635–1640.

Receptors that couple to heterotrimeric G proteins (GPCRs) are important in determining the outcome of inflammatory responses. For example, PGs that skew Th cells toward Th2-type responses act via GPCRs (1). Other examples of GPCRs important for the inflammatory responses are chemokine receptors, the receptor for the proinflammatory peptide substance P, and the β2-adrenergic receptor (2–7).

The coupling efficiency of GPCRs is tightly regulated. Recently, it has been shown that immune activation induces expression of a class of proteins called regulators of G protein signaling that attenuate G protein signaling routes (8). Another important family of proteins involved in regulation of GPCR signaling is the family of GPCR kinases (GRKs). GRKs are a family of kinases consisting of six known subtypes (GRK1–6) that play a crucial role in agonist-induced desensitization of GPCRs. Cells of the immune system express GRK2, -3, -5, and -6, of which GRK2 is the most widely studied (9–11). Interestingly, the level of GRK2 in leukocytes is 4- to 5-fold the level found in other organs such as the heart (9).

GRKs are responsible for the rapid phosphorylation of activated GPCRs that facilitates subsequent binding of regulatory proteins, β-arrestins. This process finally leads to homologous desensitization via uncoupling of the receptor from the G protein (12). GRKs and arrestins also play a key role in GPCR internalization, dephosphorylation, and recycling, thus contributing to the extent of both desensitization and resensitization of the receptors (13). The extent of agonist-induced desensitization and sequestration of GPCRs depends on the intracellular availability of GRKs and β-arrestins (14, 15). Changes in GRK expression levels alter the efficacy of GPCR signal transduction in vivo and in vitro (12, 16).

In patients with hypertension, an increase in GRK2 expression in PBMC has been reported (17). Apparently, the systemic effects of hypertension include changes in GRKs in immune cells. We have recently demonstrated that the chronic inflammatory disease rheumatoid arthritis (RA) is associated with low levels of GRKs in PBMC of these patients (18). In brain tissue from opiate addicts and in cardiac tissue from patients with chronic heart failure or myocardial ischemia, increased GRK levels have been observed (19–22). In addition, in a murine model of heart failure, the targeted cardiac overexpression of a GRK2 dominant-negative mutant can prevent the development of cardiomyopathy (23). These data suggest that changes in GRKs can contribute to the induction and/or the severity of diseases, in which GPCRs play a pivotal role. In the present study, we investigated in a rat model whether the immune-mediated disease adjuvant arthritis (AA) induces changes in GRK expression in immune and nonimmune organs. Furthermore, we tested whether changes in the levels of GRKs in the various organs are related to the severity of the disease. Specifically, we determined the level of GRK2, -3, and -6 before, at the onset, at the peak, and after recovery from the inflammatory process.

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3 Abbreviations used in this paper: GPCR, G protein-coupled receptor; AA, adjuvant arthritis; GRK, GPCR kinase; MLN, mesenteric lymph node; RA, rheumatoid arthritis.

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Materials and Methods

Animals

Inbred male Lewis rats of 6–8 wk of age were obtained from the University of Limburg (Maastricht, The Netherlands). AA was induced on day 0 by a single intradermal injection in the base of the tail with 100 μl of CFA. CFA was prepared by mixing 5 mg of heat-killed Mycobacterium tuberculosis (strain H37Ra), ground to a fine powder with 1 ml IFA. M. tuberculosis and IFA were both purchased from Difco (Detroit, MI). Rats were examined daily for clinical signs of arthritis by standard methodology (24).

Tissues and cells

On days 11, 18, 37, and 45 after arthritis induction, healthy and arthritic rats were decapitated and organs were collected. Pituitary and heart were frozen in liquid nitrogen and stored at −80°C. Immune organs (spleen, mesenteric lymph nodes (MLN), and thymus) were dispersed through filter chambers. Subsequently, splenocytes and MLN cells were isolated using Lympholite-rat (Cedarlane Laboratories, Hornby, Ontario, Canada) and Percoll (Pharmacia, Uppsala, Sweden) density gradients, respectively (25). CD4+ and CD8+ T cells and B cells were purified by magnetic cell sorting using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and mAbs specific for CD4, CD8α, and CD45RA, respectively. Thymocytes were washed once in RPMI 1640 (Life Technologies, Grand Island, NY).

GRK activity

GRK enzymatic activity was assessed using light-dependent phosphorylation of rhodopsin by cytosolic and membrane fractions from splenocytes, as previously described (18). Samples were electrophoresed on 10% SDS-PAGE. Phosphorylated rhodopsin was visualized by autoradiography. Bands corresponding to rhodopsin (~38 kDa) were quantified via liquid scintillation spectroscopy.

GRK and arrestin expression

Cell lysates were obtained by lysis in ice-cold RIPA buffer (20 mM HEPES, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AESBF), 20 μg/ml leupeptin, and 200 μg/ml benzamidine) for 30 min at 4°C. Proteins were separated by 10% SDS-PAGE (or 7.5% to assess GRK3) and analyzed for GRK and arrestin expression by immunoblotting as previously described (18). Plasma membrane fractions were used to assess GRK3, -5, and -6 since a significant amount of these kinases is associated with membranes (26–28). Immunodetection of myocardial levels of GRK2 was performed on detergent-solubilized extracts following immunoprecipitation (29). GRK2, GRK5, and GRK6 were detected using a 1/200 dilution of rabbit polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA). For GRK3, the mAb C5/1, which recognizes an epitope common to GRK2 and -3, was used at 1/5000 dilution (30). Expression of β-arrestin-1 was determined

FIGURE 1. Clinical scores during the progression of AA. Day 0 represents the time of induction with CFA. Each time point represents an average of three to six animals. Severity of arthritis was assessed by standard methodology, as described in Materials and Methods. Values represent mean ± SE.

FIGURE 2. A, GRK activity in cytosolic fractions of splenocytes from AA (n = 5, 93 ± 20 fmol/min/mg cytosolic protein) and control (C, n = 5, 309 ± 30 fmol/min/mg cytosolic protein) rats on day 18 after induction. ***, p < 0.001. Inset, Assessment of GRK activity in cytosolic fractions of splenocytes from AA and control (C) rats. The autoradiograph depicts light-dependent phosphorylation of rhodopsin (opsin, ~38 kDa). B, GRK2 immunoreactivity in splenocytes (AA, n = 5, 14 ± 3 ng/mg protein, and C, n = 5, 190 ± 12 ng/mg protein) on day 18 after induction. ***, p < 0.001. Inset, Representative Western blot depicting 5 ng of rGRK2 standard (S) and immunodetectable GRK2 in 30 μg of total cell lysate. C, GRK2 immunoreactivity in cell lysates from splenic CD4+ and CD8+ T cells, CD45RA+ B cells, on day 18 after induction. ***, p < 0.001. D, GRK2 mRNA expression assessed in splenocytes on day +18 (left panel) and on day +45 (right panel). Each lane contains 10 μg of total RNA. β-actin mRNA expression was determined on the same membrane after stripping.
using 1/500 dilution of a β-arrestin-1 mouse mAb (Transduction Laboratories, Lexington, KY). Immunoreactivity was detected by ECL (Amer sham, Buckinghamshire, U.K.). Autoradiographs were scanned using a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA).

**Northern blot analysis**

Total RNA was isolated using RNAzol-B (Campro Science, Veenendaal, The Netherlands). A total of 10 μg of RNA/lane was fractionated on a 1% agarose-formaldehyde gel and transferred to a Hybond N + membrane (Amersham). Northern blot analysis was performed as described previously (18). GRK6 mRNA expression was determined on the same filter, after stripping with 0.5% SDS, using a random primed cDNA fragment (bp 1114–2030) of GRK6. β-actin mRNA expression was determined on the same membrane using a random primed cDNA probe (1.8-kb human β-actin cDNA; Clontech, Palo Alto, CA).

**Statistical analysis**

Data are expressed as a mean value ± SE. All results were confirmed in at least two separate experiments. Specific measurements were compared using Student’s t test or one-way ANOVA, followed by Bonferroni’s analysis. Two-tailed p < 0.05 was considered to be statistically significant.

**Results**

**GRK activity and expression in splenocytes from rats with AA**

We first determined whether induction of AA results in an alteration in GRK activity in splenocytes. AA was induced by injection of rats with CFA, and disease scores were assessed. Rats did not show any signs of arthritis until day 11 after inoculation. From day 11 to day 18, there was a steep increase in the clinical score, reaching peak values at days 18–20. On day 22, inflammatory signs began to decrease until day 45, which was the last time point measured (Fig. 1). Cytosolic fractions of splenocytes were prepared from arthritic and control rats at day 18 after arthritis induction and GRK enzymatic activity was determined. Rod outer segment membranes were used as specific GRK substrate, resulting in a light (agonist)-dependent phosphorylation of the correspondent ∼38-kDa band (Fig. 2A, inset). The results demonstrated clearly that GRK activity in cytosolic fractions of splenocytes from AA rats was significantly decreased compared with splenic cytosolic samples from control rats (p < 0.001, Fig. 2A). Although GRKs are essentially cytosolic proteins, a significant amount of kinase activity is associated with the plasma membrane (31, 32). To rule out the possibility that the observed decrease in GRK activity was simply due to GRK translocation from cytosol to membrane fractions, we also assessed membrane-associated kinase activity. We found a decrease similar to that observed in cytosolic fractions (data not shown).

**GRK2 mRNA expression in spleen**

Northern blot analysis of splenic RNA was performed to analyze whether the down-regulation of GRK2 protein in AA is reflected at the mRNA level. A CDNA probe derived from human GRK2 detected a major mRNA species of ∼3.8 kb and a second minor band of ∼2.4 kb, known to be present in rat cells (Fig. 2D). Our results demonstrate that GRK2 mRNA levels in splenocytes from AA (n = 6) and control rats (n = 6) were not significantly different at day +18 after induction (GRK2: AA = 112 ± 5% of controls) (Fig. 2D, left panel). Also on day +45 after AA induction, GRK2 mRNA levels in splenocytes from AA (n = 5) and control rats (n = 5) did not differ significantly (AA = 98 ± 3% of controls) (Fig. 2D, right panel).

**GK2 protein expression in other lymphoid organs**

To assess whether the profound down-regulation of GRK2 observed in cells obtained from spleen of AA rats was also present in other lymphoid organs, we analyzed GRK2 expression in MLN cells and thymocytes. In MLN cells of AA rats at day 18 after induction of disease, GRK2 levels were reduced by 80% (p < 0.05, Fig. 3A). When CD4+ T cells, CD8+ T cells, and CD45RA− B cells separated from MLN were analyzed, the results were similar to those obtained from splenic subpopulations: GRK2 levels

**FIGURE 3.** A, GRK2 immunoreactivity in MLN cells and thymocytes on day 18 after induction in AA rats (n = 4) and controls (n = 4). *, p < 0.05. Inset A, Representative Western blot depicting 10 ng of rGRK2 standard (S) and immunodetectable GRK2 in 30 μg of whole cell lysates of MLN, or inset B, thymocytes. B, GRK2 expression in heart and pituitary from AA (n = 6) and control (n = 6) rats on day 18 after induction of AA. Inset C, Western blot of 10 ng of rGRK2 standard (S) and immunodetectable GRK2 after immunoprecipitation of GRK2 from detergent-solubilized myocardial extracts with the C5/1 Ab in control (C) and AA rats. Inset D, Western blot of 20 ng of rGRK2 standard (S) and immunodetectable GRK2 assessed in 30 μg of cytosolic fractions of pituitary from AA and control (C) rats.
were significantly decreased in CD45RA+ B cells and CD4+ T cells, whereas GRK2 levels in CD8+ T cells remained unchanged (data not shown).

Subsequently, we analyzed GRK2 expression in a primary lymphoid organ (thymus). In contrast to what was observed in spleen and MLN cells, we did not detect significant differences in GRK2 levels between thymocytes from AA and control rats on day 18 after induction (Fig. 3A and inset B).

** GRK2 protein expression in heart and pituitary **

To elucidate whether the down-regulation of GRK2 expression in AA rats was restricted to the immune system, we analyzed GRK2 expression in heart and pituitary. Myocardial levels of GRK2 protein were assessed after immunoprecipitation (Fig. 3B and inset C). Immunodetectable GRK2 in myocardial tissue from AA rats on day 18 after induction of disease does not differ significantly from GRK2 protein in myocardial tissue from control rats (Fig. 3B). Similarly, no significant differences in GRK2 protein levels were detected in pituitary cytosolic (Fig. 3B and inset D) and membrane fractions (data not shown) from AA and control rats.

** Time course of GRKs and β-arrestin protein expression in AA **

Our next question was whether the change in GRK expression levels is related to the course of the disease. Specifically, we examined splenocytes and MLN cells obtained at disease onset (day 11), at the peak of disease (day 18), and at days 37 and 45, when clinical signs of inflammation had almost disappeared (Fig. 1). On days 11 and 18 after AA induction, we observed a marked reduction in GRK2 protein levels (up to ~80–85%) in spleen and MLN cells. On day 37 after induction of AA, GRK2 protein levels in spleen cells are still very low, whereas GRK2 levels in MLN cells had returned to the level of control rats. At day 45, GRK2 levels in splenocytes had increased significantly (p < 0.01 vs day 18), but were still lower than in control rats (~50% decrease, p < 0.05) (Fig. 4A). In thymocytes and heart, no changes in GRK2 expression levels were detected at days 11, 37, and 45 (data not shown).

Apart from GRK2, lymphoid cells also express three other subtypes of GRK (GRK3, GRK5, and GRK6) (9, 11, 33). Immunodetectable GRK3 was decreased by ~45% (p < 0.05, Fig. 4B and inset) in splenocytes from AA rats on day 18 after induction and had returned to baseline level at day 37 (Fig. 4B). GRK5 was barely detectable in plasma membranes from splenocytes of healthy and AA rats (data not shown). Immunodetectable GRK6 in splenocytes was reduced profoundly by ~80% (p < 0.01) in AA rats on days 11 and 18 and was back to normal on day 37 (Fig. 4C and inset). Similarly to GRK2, the profound down-regulation of GRK6 expression levels was not mirrored by a down-regulation at the mRNA level (GRK6: AA = 103 ± 4% of controls at day +18).

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** FIGURE 4. **A. Time course of immunodetectable GRK2 assessed in splenocytes (SPLC) and MLN cells (MLNC). Data are arbitrary density units expressed as percentage of control values (mean of control value = 100). Three to six animals for each time point were examined. B, Time course of GRK3 immunoreactivity in plasma membrane of splenocytes from AA (n = 4) compared with control (n = 4) rats. *, p < 0.05 vs controls. Insert, Representative immunoblot depicting 10 ng of rGRK2 and 10 ng of rGRK3 standard (S), and immunodetectable GRK2/3 assessed in 30 μg of plasma membrane protein of splenocytes from AA and control (C) rats on day 18 after induction. C, Time course of GRK6 immunoreactivity in plasma membrane of splenocytes. Data are arbitrary density units expressed as percentage of control values (mean of control value = 100). Three to six animals for each time point were examined. ***, p < 0.01 vs controls. Insert, A representative Western blot depicting rGRK6 standard (S) and immunodetectable GRK6 assessed in 15 μg of plasma membrane protein of splenocytes from AA and control (C) on day 18 after induction. D, Time course of β-arrestin-1 immunoreactivity in whole cell lysate of splenocytes from AA and control (C) on day 18 after induction. Insert, A representative Western blot depicting immunodetectable β-arrestin-1 assessed in 30 μg of whole cell lysates of splenocytes from AA and control (C).
Next, we investigated whether AA was associated with changes of the GRK cofactor β-arrestin-1. In splenocytes from AA rats on day 18 after induction of the disease, we observed a moderate, but statistically significant, increase in immunodetectable β-arrestin-1 (Fig. 4D and inset) compared with controls (AA = 136 ± 5% of expression in controls on day 18, p < 0.01). On day 45, β-arrestin-1 levels in splenocytes had returned to the levels of control rats (Fig. 4D and inset). A similar time course for β-arrestin-1 expression was obtained in MLN cells (data not shown).

Discussion
GRKs play a crucial role in the regulation of a vast array of GPCRs involved in neurotransmission, cardiovascular function, and immune response, including inflammatory processes (13). The results presented in this work clearly demonstrate that the inflammatory disease AA induces tissue-specific alterations in the expression and activity of GRKs. During AA, expression of GRKs is altered in secondary immune organs. Interestingly, sustained activation of β2-adrenergic receptors by infusion of isoproterenol for 14 days in rats induces changes in GRK2 levels in heart as well as in PBMC (34). Moreover, spontaneously hypertensive rats show a significant increase of immunodetectable GRK2 both in lymphocytes and vascular myocytes when compared with normotensive rats (35). Based on these data, we expected to find alterations in GRKs during arthritis not only in immune organs, but also in nonimmune organs. However, although AA is a systemic inflammatory disease, the changes in GRK levels were restricted to secondary immune organs (Figs. 2 and 3). No change in GRKs was observed in a primary immune organ (thymus) or in heart and pituitary of arthritic rats (Fig. 3). Our results demonstrate that down-regulation of GRKs only occurs at the site in which the immune response takes place. In this respect it is of interest that only subsets are involved in arthritis, namely CD4+ T cells and B cells (36–38), display decreased GRK2 levels. In contrast, GRK2 remains unchanged in CD8+ T cells that are not required for expression of the disease in animal models (37, 38).

Down-regulation of GRK2 protein level in immune cells can be induced in vitro by proinflammatory cytokines, e.g., IL-6 and IFN-γ (18). Although receptors for proinflammatory cytokines are present on many tissues, including thymus, pituitary, and heart (39–41), and circulating levels of cytokines are increased in arthritis (42), GRK levels in these nonimmune organs are unaltered. Thus, if cytokines are responsible for GRK decreases in immune organs of arthritic rats, one has to assume that this effect of cytokines is limited to activated immune organs.

Previously, we reported that PBMC of humans with RA express lower levels of GRK protein without changes in GRK mRNA (18). In the present study, we demonstrate that a similar phenomenon occurs in rat splenocytes during AA. The observation that mRNA levels for the GRKs do not change during arthritis suggests that the down-regulation of GRKs involves a process of posttranscriptional modification and/or protein degradation. Nonspecific degradation of proteins can be excluded since the level of β-arrestin-1 does not decrease, but rather increases during AA (Fig. 4D). Penela et al. (43) recently described that β2-adrenergic receptor stimulation enhances GRK2 degradation by the proteasome pathway. It may well be possible that in arthritis mediators that signal through GPCR, for example chemokines and catecholamines, enhance GRK2 degradation via proteasomes through stimulation of their respective receptors (44, 45). In addition, we have preliminary evidence that activated granulocytes can induce a down-regulation of GRK2 in PBMC of healthy individuals via the production of oxygen radicals (M.S.L., A.K., L. Scholtens, M. Roccio, M.S., R.E.S., and C.J.H., manuscript in preparation). Oxygen radicals are produced in high amounts during inflammatory processes by activated granulocytes and macrophages and are potent activators of the proteasome pathway (46, 47). Therefore, it is tempting to hypothesize that in activated immune organs, oxygen radicals are responsible for local degradation of GRK2 protein. If this hypothesis is correct, the normal GRK2 levels in heart, pituitary, and thymus of arthritic rats can be explained by the absence of activated granulocytes or macrophages in these organs during the disease process.

At the peak of AA in rats, we also observed a profound down-regulation of GRK6 and, to a lesser extent, of GRK3 in spleen and MLN cells (Fig. 4). At the same time, the expression of β-arrestin-1, another protein involved in the desensitization/resensitization of GPCRs, is actually increased (Fig. 4D). During the remission phase of arthritis, GRK2 and GRK6 levels in spleen have partially or completely returned to baseline levels. The fact that changes in GRK levels develop after induction of arthritis in rats and return to normal levels during the remission phase suggests that inflammatory activity determines how much GRK will be present in immune cells. Our present results in arthritic rats also suggest that in patients with RA, the low GRK2 levels are the consequence of the disease process and do not reflect preexisting low levels of GRK2 in these patients.

One of the functional effects of down-regulation of GRKs can be an increased sensitivity of various GPCRs in vivo. We have demonstrated that PBMC of RA patients, showing a ∼50% reduction in GRK2 expression and activity, are more sensitive to β2-adrenergic activation (18). Furthermore, we have preliminary evidence that splenocytes of AA rats have an enhanced response to triggering of the PGE2 receptor, that is a substrate for GRKs. More direct evidence for the physiological importance of GRK2 levels has been obtained by expression of a GRK2 dominant-negative mutant in cells that also express the chemokine receptor CCR2B. In those experiments, it has been shown that the dominant-negative mutant prevents homologous desensitization of the CCR2B receptor after exposure to the agonist monocyte chemoattractant protein 1 (44). Conversely, overexpression of GRK2 has been shown to enhance the desensitization of the opiate receptor (48). Finally, in heterozygous GRK2+−/− knockout mice, the low GRK2 activity is associated with increased in vivo contractile responses to the β2-adrenergic agonist isoproterenol (49, 50). Based on these data, we would like to propose that down-regulation of GRKs during (chronic) inflammation may lead to increased or sustained activation of G protein-coupled proinflammatory receptors, e.g., chemokine receptors.

In conclusion, we demonstrated in this study, for the first time, that an inflammatory process in vivo induces tissue and cell type-specific down-regulation of GRKs in immune organs involved in the inflammatory response. Therefore, research aimed at specific regulation of GRKs may lead to new therapeutic strategies on the level of receptor regulation in chronic inflammatory diseases, such as rheumatoid arthritis.

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