Hypoxia/Ischemia Modulates G Protein–Coupled Receptor Kinase 2 and β-Arrestin-1 Levels in the Neonatal Rat Brain

Maria Stella Lombardi, PhD; Evelyn van den Tweel, MSc; Annemieke Kavelaars, PhD; Floris Groenendaal, MD, PhD; Frank van Bel, MD, PhD; Cobi J. Heijnen, PhD

Background and Purpose—Neurotransmitters, neuropeptides, chemokines, and many other molecules signal through G protein–coupled receptors (GPCRs). GPCR kinases (GRKs) and β-arrestins play a crucial role in regulating the responsiveness of multiple GPCRs. Reduced expression of GRK and β-arrestins leads to supersensitization of GPCRs and will thereby increase the response to neuropeptides and neurotransmitters. We analyzed GRK and β-arrestin expression after cerebral hypoxia/ischemia (HI).

Materials and Methods—Twelve-day-old rat pups were exposed to 90 minutes of hypoxia (fraction of inspired oxygen [FiO2] 0.08) after ligation of the right carotid artery, a procedure that induces unilateral damage in the right hemisphere. At 6, 12, 24, and 48 hours after HI, the left (hypoxic) and right (hypoxic/ischemic) hemispheres were analyzed for GRK and β-arrestin protein and mRNA expression by Western blotting and real-time polymerase chain reaction, respectively. In addition, we analyzed GRK2 expression in the hippocampus by immunohistochemistry.

Results—HI downregulated GRK2 protein expression in both hemispheres at 24 to 48 hours after HI, and the effect was more pronounced in the ipsilateral hemisphere. HI induced no global change in GRK6 protein expression. However, GRK2 was markedly decreased in the hippocampal region of the ipsilateral hemisphere that will be severely damaged after HI. No changes in global mRNA levels for GRK2 were detected. In contrast, HI increased β-arrestin-1 protein expression as well as mRNA levels at 6 to 12 hours after HI.

Conclusions—Neonatal HI-induced brain damage is associated with specific changes in the GPCR desensitization machinery. We hypothesize that these changes result in supersensitization of multiple GPCRs and might therefore contribute to HI-induced brain damage. (Stroke. 2004;35:000-000.)

Key Words: hypoxia ■ ischemia ■ newborn ■ receptors, G-protein coupled

Perinatal cerebral hypoxia/ischemia (HI) is one of the most common causes of neonatal mortality and morbidity. Long-term effects include cerebral palsy, mental retardation, and epilepsy.1 During HI the lack of oxygen and nutrients initiates a complex cascade of events, ultimately leading to neuronal cell death. Multiple pathways involved in neuronal injury have been described, such as elevation of extracellular glutamate and activation of glutamate receptors, with a subsequent generation of reactive oxygen species and nitric oxide.2 In addition, it has been shown that perinatal HI with reperfusion rapidly triggers acute inflammatory responses in the brain, including cytokine3 and chemokine4 production, that contribute to neuronal and glial cell death. In the interplay of signaling pathways activated during HI, G protein–coupled receptors (GPCRs) play a pivotal role. GPCRs represent the largest family of membrane receptors capable of conveying information from a wide variety of neurotransmitters, hormones, neuropeptides, and chemokines.5 Many functional processes in the brain are affected via receptors of the GPCR family, including α- and β-adrenergic, dopaminergic, muscarinic, metabotropic glutamate, serotonergic, and chemokine receptors. Some of these receptors play an important role in the pathology of HI stress as well as in normal postnatal brain development.6,7 Therefore, tight regulation of the sensitivity of this class of receptors is an important feedback mechanism capable of limiting both acute and chronic overstimulation of GPCR signaling.

The sensitivity of GPCRs is regulated by the so-called G protein–coupled receptor kinases (GRKs). So far, 7 members of the GRK family have been cloned (GRK1 through 7).8 Although some studies with overexpression systems have suggested that GRK isoforms are functionally redundant with respect to desensitization of the GPCR, the recent generation of knockout mice models has shown that there is a high degree of GRK-GPCR specificity.9 GRKs promote homologous receptor desensitization by phosphorylating agonist-bound GPCR, thereby allowing binding of another family of proteins, termed β-arrestins, which inhibit further receptor/G-protein coupling and terminate the response to the ligand. Moreover, binding of β-arrestins promotes receptor internal-
ization and is involved in GRK degradation as well as intracellular signaling.\(^\text{10}\) Importantly, the intracellular level of GRK and β-arrestins determines the relative ability of a given receptor to desensitize.\(^\text{11}\) GRK and β-arrestins are ubiquitously expressed, and the expression of GRK2, GRK6, and β-arrestin-1 is particularly high in the brain and the immune system.\(^\text{9}\) Changes in GRK expression contribute to pathologic states in which GPCRs play a pivotal role. GRK2 expression levels are increased in hypertension,\(^\text{12}\) heart failure,\(^\text{13}\) and cardiac hypertrophy experimental models.\(^\text{14}\) Moreover, we described a marked downregulation of GRK2 protein expression in leukocytes during inflammatory autoimmune diseases.\(^\text{15}\) GRK levels can be (down)modulated by cytokines like interleukin-6 and interferon-γ,\(^\text{15,16}\) as well as by oxidative stress.\(^\text{17}\) Interestingly, in leukocytes the breakdown of GRK2 protein after oxidative stress appears to be mediated by the protease calpain.\(^\text{17}\) Because we know that HI increases expression of proinflammatory cytokines, oxygen radicals, and calpain in the brain, we examined the expression of components of the GRK/arrestin machinery after HI in neonatal rats.

### Materials and Methods

#### Hypoxia/Ischemia

The Experimental Animal Committee of UMC-Utrecht approved all experimental protocols. At postnatal day 12, as a model for the full-term human neonate, Wistar rat pups of both sexes were exposed to HI.\(^\text{18}\) Animals were anesthetized with halothane (5% induction, 2% maintenance) in N\(_2\)O/O\(_2\) (1:1, vol/vol). The right common carotid artery was ligated. After 60 minutes, animals were exposed to 8% O\(_2\)/92%N\(_2\) (90 minutes, 37°C). Pups were returned to their dams and decapitated at 6, 12, 24, or 48 hours after HI. Sham controls underwent surgery without artery occlusion and were humanely killed 24 hour later. To assess the effect of hypoxia only, naive animals were or were not exposed to hypoxia, as indicated previously. Brain hemispheres were stored at −80°C.

#### Western Blotting

Brain hemispheres were homogenized as described before.\(^\text{19}\) Proteins (40 μg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond-C, Amersham UK) by electroblotting. Blots were stained with GRK2 (Sc-562) and GRK-6 (Sc-566) antibodies (Santa Cruz Biotechnology) or β-arrestin-1 mouse monoclonal antibody (Becton Dickinson) and developed with enhanced chemiluminescence (Amersham). Band density was determined with a GS-700 densitometer (Bio-Rad).

#### Immunohistochemistry

Forty-eight hours after HI, animals were sedated (pentobarbital 50 mg/kg, intraperitoneal) and intracardially perfused with 4% paraformaldehyde, and the brains were removed, postfixed, and embedded in paraffin.\(^\text{20}\) Eight-micron-thick coronal sections were stained with anti-GRK2, horseradish peroxidase–conjugated goat anti-rabbit antibody (Biotin), and Vectastain-ABC (Vector Laboratories).

#### Real-Time PCR

Total RNA was extracted with Trizol reagent (Life Technologies). cDNA was synthesized from 0.5 μg total RNA with the Superscript RNase H-reverse transcriptase kit (Invitrogen). Oligonucleotide primers (Table 1) were from TibMolbiol. Real-time polymerase chain reaction (PCR) was performed with use of DNA Master SYBR-green kits and a LightCycler apparatus (Roche Applied Science). Melting curve analysis and agarose gel electrophoresis confirmed amplification specificity. 18s rRNA was used for data normalization.

### Results

#### GRK Protein Expression

Protein levels of GRK2 and GRK6 in total lysates from the ipsilateral and contralateral hemispheres at 6, 12, 24, and 48 hours after HI induction were analyzed. The HI ipsilateral hemispheres show a decrease in GRK2 expression by ≈40% at 6 hours (P<0.001 vs sham), which reached an ≈70% decrease by 24 to 48 hours (P<0.001; Figure 1A). In the contralateral hemispheres, an ≈55% GRK2 downregulation was induced that became evident only at 24 and 48 hours after HI (P<0.01 vs sham). The differences between ipsilateral and contralateral hemispheres were significant at 6 and 24 hours (trend at 12 hours). We also analyzed the effect of hypoxia only; however, no significant changes were detected for GRK2 protein expression at 6 to 48 hours after hypoxia alone (Figure 1C). The changes in GRK2 protein expression cannot be attributed to a developmental effect, because in control animals GRK2 expression was already increased at 6 hours after HI (P<0.001 vs sham), and returned to control levels by 48 hours.

#### β-Arrestin-1 Protein Expression

In the ipsilateral hemispheres β-arrestin-1 protein, a cofactor of GRK2, was already increased at 6 hours after HI (P<0.01), reached a maximal fourfold increase at 12 hours after HI (P<0.001 vs sham), and returned to control levels by 48 hours.

In the contralateral hemisphere, β-arrestin-1 levels tended to increase at 6 hours after HI (P=0.054), significantly increased at 12 to 24 hours (P<0.01 vs sham), and also returned to normal levels by 48 hours after HI. No significant differences were detected between ipsilateral and contralateral hemispheres (Figure 2A). No changes in β-arrestin-1 expression were detected after hypoxia alone (Figure 2B). Similar to what was observed for GRK2, we did not detect any change in β-arrestin-1 expression at postnatal days 12 through 14 (data not shown).

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>GRK2</td>
<td>Fw5′ − AAACGGAAGCTCGCCAGA</td>
<td>Rev5′ − GCACAGCTCAGGGTCACTAT</td>
</tr>
<tr>
<td>GRK6</td>
<td>Fw5′ − GCTAGAACACCTTTAAAACTTG</td>
<td>Rev5′ − TCACCACATCCTGGTCTGCC</td>
</tr>
<tr>
<td>β-Arrestin-1</td>
<td>Fw5′ − CCACATCGGAGAATCTCGAGA</td>
<td>Rev5′ − CAGCGGCTGACGAGCAA</td>
</tr>
<tr>
<td>18s rRNA</td>
<td>Fw5′ − GTACCCGTTGAAACCCCATT</td>
<td>Rev5′ − CCATCAATCGGTA6T6CGG</td>
</tr>
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</table>

Fw indicates forward; rev, reverse.
Immunohistochemistry

Immunohistochemical analysis at 48 hours after HI revealed an overall decrease in GRK2 in the ipsilateral cortex and hippocampus (Figure 3A). A detailed analysis of the hippocampal area showed that GRK2 was predominantly expressed in neuronal cells (Figure 3C through 3E and data not shown). At 48 hours after HI, there was a slight reduction in GRK2 in the hippocampus and dentate gyrus in the contralateral hemisphere (Figure 3D and 3E). Interestingly, GRK2 was markedly lower in the ipsilateral hemisphere versus the contralateral hemisphere (Figure 3D and 3E). The reduction in GRK2 cannot be attributed completely to neuronal cell death, because this trend was also observed in intact ipsilateral neuronal cells (insets of Figure 3C and 3D). Immunohistochemistry for GRK6 and β-arrestin-1 could not be performed due to a lack of suitable antibodies.

GRK and β-arrestin-1 mRNAs

Global GRK2 mRNA expression in the contralateral or ipsilateral hemisphere of HI rats was not different from GRK2 mRNA levels in sham controls (Figure 4A). Analysis of GRK6 mRNA expression, however, revealed a 5- to 6-fold increase in both hemispheres at 6 and 12 hours after HI compared with sham control animals, which normalized to sham values at 24 and 48 hours after HI (Figure 4B). For β-arrestin-1 in both hemispheres at 6 to 12 hours after HI, a 6- to 7-fold increase in mRNA (P<0.05) was detected. β-Arrestin-1 levels returned to control levels at 24 to 48 hours after HI (Figure 4C).
Discussion

We demonstrate here for the first time that HI reduces the global expression of brain GRK2, a key enzyme involved in regulating the responsiveness of many GPCRs. Moreover, the GRK2 cofactor β-arrestin-1 was increased in the brain after neonatal HI when compared with sham-operated animals. It is not likely that the decrease in GRK2 expression can be explained completely by cell death, because colocalized β-arrestin-1 was not decreased, and viable neuronal cells in the ipsilateral hippocampus after HI express reduced GRK2 levels.

Even modest changes in the endogenous level of GRK2 and β-arrestin-1 can alter the extent of receptor desensitization and internalization.\textsuperscript{11} Regulation of the level of intracellular GRK2 and β-arrestin-1 might therefore represent a crucial event in the response of the brain in situations of excessive or prolonged availability of ligands after HI. In heterozygous GRK2-knockout mice, which show a 50% reduction in GRK2 protein expression, the sensitivity of the GRK2 substrate CCR5 is increased, leading to increased chemotaxis.\textsuperscript{20a} Furthermore, a 50% reduction in cardiac GRK2 levels is associated with a significant increase in the contractile response of the heart to a β-adrenergic agonist.\textsuperscript{14} The downregulation of GRK2 in HI is particularly important in light of the sensitivity of metabotropic glutamate receptors (mGluRs), which are involved in excitotoxic damage. GRK2 has been shown to desensitize both constitutive and agonist-acti-
vated mGluR1-mediated cellular responses. The activation of mGluR1 not only increases glutamate release but also potentiates N-methyl-D-aspartate (NMDA) receptor–induced neuronal cell death. Therefore, a decrease in GRK2 might increase not only mGluR1-mediated cell death but also NMDA receptor–mediated neuronal damage. Interestingly, despite the fact that in vitro multiple GRKs can phosphorylate 1 receptor, the recent generation of knockout mouse models suggests a high degree of GRK specificity in vivo. For example, in vitro both GRK2 and GRK3 phosphorylate β-adrenergic receptors. However, in vivo a 50% decrease in GRK2 results in increased β-adrenergic receptor–mediated cardiac contractility, suggesting that GRK3 cannot compensate for reduced GRK2 in vivo. Similar GRK receptor specificity has been described in vivo for the leukotriene B4 receptor and GRK6 or the muscarinic receptor and GRK5. Therefore, we conclude that in vivo, changes in the level of 1 given GRK will really lead to a change in the response of specific substrate receptors.

The developmental pattern of GRK2 and β-arrestin-1 expression in the rat brain shows an increase during the second and third week after birth, which suggests a functional involvement of these proteins in brain development during this particular period. In view of this evidence, we hypothesize that alterations of GRK2 and β-arrestin-1, as induced by HI during early life, not only might have important consequences for direct perinatal brain damage but might also interfere with the maturation process of the neonatal brain. In the present study the 12-day-old rat was chosen, because the developmental stage of the brain reflects that of a full-term human neonate.

GRK2 downregulation is observed in both hemispheres, suggesting that hypoxia is responsible for decreased GRK2 in the contralateral hemisphere. However, hypoxia only does not induce changes in GRK2 and β-arrestin-1, implying that additional mechanisms are responsible for the change in GRK2 and β-arrestin-1 in the contralateral hemisphere, which should be either of neuronal or blood-borne origin. The phenomenon of ipsilateral and contralateral effects of HI has also been shown in other studies with respect to the expression of cytokine interleukin-1β or phosphorylated extracellular receptor kinase (pERK1/2).

The decrease in GRK2 was more pronounced and the kinetics of downregulation were different as well, starting at 6 hours in the ipsilateral side in comparison with 24 hours on the contralateral side. We showed earlier that neuronal damage in the hippocampus after 6 weeks occurs only in the ipsilateral hemisphere. We speculate therefore that the early downregulation of GRK2 is more important than the late downregulation that occurs in both hemispheres. Another perhaps more likely explanation is that low GRK2, resulting in a higher sensitivity of GPCRs involved in the process of neonatal brain damage, is only leading to actual damage when the availability of the ligands in the ipsilateral hemisphere is increased, as has been shown for glutamate.

HI downregulates GRK2 without inducing changes in global GRK2 mRNA levels. Although we cannot exclude the possibility that there are subtle local changes in GRK2 mRNA expression that might have been masked in our analysis of the whole hemisphere, it is likely that the downregulation of GRK2 protein occurs at the posttranscriptional level. GRK2 can be degraded via the proteasome pathway, and its turnover is increased by GPCR stimulation, which will occur after HI. In addition, HI is accompanied by an increase in cytokine expression in the brain. We have demonstrated earlier that cytokines like interleukin-6 and interferon-γ downregulate GRK2 in leukocytes. Therefore, we propose that the increase in cytokine expression in the

Figure 4. GRKs and β-arrestin-1 mRNA expression after HI. Real-time PCR analysis of GRK2 (A), GRK6 (B), and β-arrestin-1 (C) mRNA expression after HI. Brains were collected at different time points after HI (n=6 per group) and used for real-time PCR analysis as described in Methods. The levels of expression for each target were measured and normalized against 18S rRNA. *P<0.05, **P<0.01 compared with sham.
brain will contribute to GRK2 downregulation. We previously
demonstrated in leukocytes that the breakdown of GRK2 after
oxidative stress in vitro is prevented by the calpain inhibitor
calpeptin.17 Therefore, HI-induced increase in the protease
calpain might also contribute to GRK2 breakdown in the brain.

Global protein expression of GRK6 was not changed. However,
global mRNA for GRK6 increased by 6- to 7-fold at 6 to 12 hours
after HI. This discrepancy between mRNA and protein levels
suggests that the mRNA is not translated. However, because we
could not perform immunohistochemistry, we cannot exclude the
possibility that the global increase in GRK6 mRNA was associated
with specific regional increases in GRK6 protein. We observed an
increase in β-arrestin-1, a cofactor that is involved in GRK2-
dependent receptor internalization. Recently, it has been shown that
β-arrestin-1 can also serve to facilitate GRK2 degradation via a
specific ERK1/2 after neonatal rat cerebral hypoxia-ischaemia.

In conclusion, we have shown that HI leads to decreased
GRK2 and increased β-arrestin-1, intracellular proteins that
regulate the sensitivity of many GPCRs in the brain. We
hypothesize that increased cytokines, calpain, and/or
β-arrestin-1 in the brain are involved in the process of GRK2
degradation. Because one pivotal kinase like GRK2 might be
powerful enough to influence many injurious processes that
occur after HI, it will be obvious that short-lasting therapies to
prevent GRK2 degradation should be considered as a novel
target for the reduction of HI-induced neonatal brain injury.

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