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D₅ dopamine receptor regulation of reactive oxygen species production, NADPH oxidase, and blood pressure

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Submitted 20 June 2005; accepted in final form 12 September 2005

DURING THE PAST DECADE, DOPAMINE has been shown as an important regulator of blood pressure, sodium balance, and renal and adrenal function through an independent peripheral dopaminergic system (32). Dopamine exerts its actions via two families of cell surface receptors that belong to the superfamily of G protein-coupled receptors. D₁-like receptors (D₁ and/or D₅) stimulate adenylyl cyclases, while D₂-like receptors (D₂, D₃, and D₄) inhibit adenylyl cyclases (32, 45). Abnormal signaling of D₁-like receptors has been shown to be involved in rodent models of genetic hypertension and in humans with essential hypertension (7, 10, 17, 28, 32, 55). However, the precise D₁-like receptor involved remains to be determined. There is an abnormal renal D₁ function in hypertension, which is caused by activated variants of the G protein-coupled receptor kinase type 4 (16, 32). The D₅ locus is not linked to hypertension in Dahl salt-sensitive rats (22), and mutations of the D₅ are not found in spontaneously hypertensive rats (3). However, the locus of the human D₅ (hD₅), 4p15.1–16.1 (1, 9), and its pseudogenes, lq21.1 and 2p11.1-p11.2 (23), have been linked to human essential hypertension (13, 14). Moreover, humans have D₅ gene single nucleotide polymorphisms with diminished function (15). Indeed, disruption of the D₅ gene in mice (D₅−/−) causes hypertension (27). However, the mechanism(s) responsible for the increase in blood pressure in D₅−/− mice remains to be determined.

The production of reactive oxygen species (ROS) was first discovered as part of the host defense system of phagocytes (47). In addition to mediating intracellular killing of pathogens in leukocytes and macrophages, ROS have also been shown to be important mediators of cell injury in various pathophysiological conditions, such as aging, ischemia, and hypertension (6, 20, 33, 56). ROS encompass a series of oxygen intermediates that include the superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (HO·), and hypochlorous acid. A major source of O₂⁻ in vascular tissues and kidney is NADPH oxidase, an enzymatic complex that consists of six subunits: membrane subunits p22phox and gp91phox, cytosolic components p40phox, p47phox, and p67phox, and a low-molecular-weight G protein Rac1 or Rac2 (4, 18, 41, 49, 51). Several homologs of gp91phox in nonphagocytic tissues, organs, and cell lines have been cloned, including the Nox (NADPH oxidase) subgroup (Nox 1, Nox 2, Nox 3, Nox 4, Nox 5) and the Duox enzymes (Duox 1 and Duox 2) (21, 41, 49, 51). Interestingly, Nox 4 has been shown to act as an O₂⁻-producing NADPH oxidase in vascular smooth muscle and kidney (21, 49).
D1 and D3 have been shown to inhibit oxidative stress in vascular smooth muscle cells through activation of PKA and suppression of phospholipase D (PLD) and PKC (60). Furthermore, we recently found that the D3 stimulation inhibits PLD2 activity (59), the product of which activates NADPH oxidase.

In the present study, we examined the effect of and the mechanism by which the D3 activation regulates ROS production. The role of D3-mediated regulation of ROS on blood pressure was tested in mice in which the D3 gene (D3/−/−) was disrupted (27, 59). Our studies focused on the brain and the kidney because hypertension in D3/−/− mice is, in part, caused by activation of oxytocin, V1 vasopressin, non-NMDA, and α-adrenergic receptors in the central nervous system (27), and the kidney plays an important role in the long-term regulation of blood pressure (25). The cellular mechanism by which the D3 activation regulates ROS production was also studied in HEK-293 cells heterologously expressing the human D3.

**MATERIALS AND METHODS**

**D3/−/− Mice and Blood Pressure Measurement**

The generation of D3/−/− mice has been reported previously (27). In our studies, we used >F2, generation mice in a C57BL/6 background (3–6 mo old) (>98% congenic). C57BL/6 mice are less susceptible than 129/SV mice to the development of DOCA-induced hypertension and renal damage (26) and global cerebral ischemic injury (19). D3+/+ littermates were used as controls. Our protocol was reviewed and approved by the Georgetown University Animal Care and Use Committee.

Anesthetized blood pressures were measured from the aorta, via the femoral artery. The mice were anesthetized with pentobarbital sodium injected intraperitoneally at 50 mg/kg body wt, placed on a heated board to maintain a 37°C body temperature, and tracheotomized with a polyethylene (PE)-90 catheter. A catheter (PE-50 heat-stretched to 180-μm tip) was then inserted into the right femoral artery and pushed toward the aorta to monitor blood pressure. Blood pressures were monitored using Cardiomax II (Columbus Instruments, Columbus, OH) (27, 59).

The blood pressures of conscious >F2 D3 mice with a modest sodium diet (4% NaCl) were monitored by telemetry. The transmitter body of the device was secured inside a skin pocket fashioned on the right flank of the mice. The skin was sutured, and the mice were closely observed and kept warm until recovery. An analgesic dose of buprenorphine (2.5–3.0 mg/kg) was given subcutaneously or intraperitoneally to alleviate any postoperative pain. The mice were subsequently transferred to their original shoebox cage, which was then placed over the receiver board. The computer Datquest enabled the continuous monitoring and recording of all data at predetermined intervals. Only the data obtained after the mice had recovered with a full return to normal activity (about 7 days) were analyzed. The mice were fed a normal NaCl diet (0.8%) for 14 days and then changed to a modestly increased NaCl diet (4%) for 15 days.

On the basis of the conscious blood pressure studies, we studied the effect of apocynin (Sigma, St. Louis, MO), a drug that inhibits NADPH oxidase activity by impeding the assembly of the p47phox and p67phox subunits with the membrane NADPH oxidase complex (52). Mice were fed with 4% NaCl diet for 15 days and then treated with apocynin (1 mmol kg body wt \textsuperscript{-1} day \textsuperscript{-1}) or vehicle (saline, control) intraperitoneally for 10 days. Therefore, blood pressures were measured from the aorta via the femoral artery in anesthetized mice (27, 59). The kidneys and brains were harvested and immunoblotted for gp91phox and Nox 4, and then NADPH oxidase activity was quantified. Plasma thiobarbituric acid-reactive substances (TBARS) were also measured as an index of systemic oxidative stress.

**Cell Cultures and Transfections**

A full-length human (h) D3 (hD3) cDNA was subcloned into a pcDNA6/V5-His vector between the EcoRI and XhoI sites (59) and was transfected into HEK-293 cells (HEK-hD3 cells) using LTX transfection reagents (Mirus, Madison, WI). Stably transfected single colony was selected with 10 μg/ml blasticidin. The successful transfection of hD3 cDNA was verified by immunoblotting for His/V5 expression and by measuring CAMP production as an indicator of hD3 function. In some HEK-293 cells, the catalytically inactive variant of PLD2, K758R-PLD2, was cotransfected into HEK-hD3 cells (44, 59). Empty vector-transfected HEK-293 cells served as negative controls.

**Immunoblotting**

Mouse kidney homogenates and membranes or cells were prepared for immunoblotting, according to our previously published procedure (59). Fifty to one hundred micrograms of the samples were immunoblotted with well-characterized monoclonal anti-human gp91phox and p47phox antibodies (4), and anti-Nox 4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Uniformity of protein loading and membrane transfer were determined by immunoblotting for β-actin.

**Analysis of ROS Production**

Fifteen micrograms of membrane proteins from HEK-293 cells and mouse kidney/brain tissues were prepared, and NADPH oxidase activity (light units per microgram protein per minute) was determined by measuring NADPH-induced chemiluminescence in the presence of lucigenin (5 μmol/l) and NADPH (100 μmol/l) (ICN Biomedicals, Seoul, South Korea) (50). The specificity of the NADPH-dependent O2\textsuperscript{−} production was verified by treatment with diphenylene iodonium (DPI) (Sigma), a flavoprotein inhibitor.

O2\textsuperscript{−} production was measured in HEK-293 cells using a cytochrome c assay in the presence of NADPH (100 μmol/l), and cytchrome c reduction was monitored at 550 nm (35). Controls also contained superoxide dismutase (SOD, 300 U assay) (Sigma). To determine whether PKA was involved in the pathway, cells were pretreated with PKA inhibitors, H-89 (Calbiochem), Rp-cAMPS, and Rp-8-Piperidino-cAMPS (Biolog Life Science Institute, Hayward, CA) and adenylyl cyclase inhibitor SQ22536 (Biomol Research Laboratories, Plymouth Meeting, PA), before the addition of the D1-like receptor agonist, fenoldopam (Sigma). Because HEK-hD3 cell heterologously express the D3 but not the D1 receptor, fenoldopam behaves as a D3 agonist in our system. The following equation was used to calculate O2\textsuperscript{−} production: \text{nmol O2\textsuperscript{−}/10\textsuperscript{6} cells = [A550 (without SOD) − A550 (in the presence of SOD)] × 159.}

H2O2 production was measured in HEK-293 cells by monitoring homovanillic acid (ICN Biomedicals) oxidation in phenol red-free Hank’s balanced salt solution containing 5 U/ml horseradish peroxidase type VI (Sigma) with or without catalase (20 U/ml) (Calbiochem). The assay mixture was read in a fluorometer (excitation 321 nm, emission 421 nm) with a slit of 1 cm at 25°C. The H2O2 produced was expressed as nmol h\textsuperscript{-1}10\textsuperscript{6} cells\textsuperscript{-1} (calibrated with H2O2) (54).

**Measurement of TBARS**

TBARS in plasma of D3 mice were measured as malondialdehyde (MDA) equivalents (μmol MDA/l plasma), according to a published protocol (40). After the apocynin study on blood pressures in anesthetized D3 mice, D3 mouse blood was collected into heparin (100
units/1.0 ml blood)-containing test tubes, and centrifuged. Plasma (160 μl), mixed with 2% butylated hydroxytoluene and quinlanilla reagent, was boiled for 15 min. After the reaction mixtures were cooled, the samples were centrifuged (3,000 g for 10 min), and spectrophotometric readings (535 nm) were obtained. The readings were compared with MDA standards (Sigma). MDA levels were taken as an index of systemic oxidative stress.

Statistical Analysis

Data are expressed as means ± SE. Comparison among and within groups was made by factorial and repeated-measures ANOVA and Scheffe’s (multiple comparison) or Dunnett’s (vs. control) tests, and a comparison between two groups used the Student’s t-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

In Vivo Studies

Blood pressures in D5 mice. Analysis of blood pressure demonstrated that conscious D5−/− mice were hypertensive, while D5+/+ mice were normotensive. The increased blood pressure in D5−/− mice is consistent with our previous pentobarbital-anesthesia studies on blood pressure (27, 59). The blood pressures in D5−/− mice increased further after 15 days of an oral sodium load (4% NaCl) (Fig. 1A).

The high blood pressures, measured under pentobarbital sodium anesthesia in D5−/− mice, were normalized after 10 days of intraperitoneal apocynin, an NADPH oxidase inhibitor, whereas apocynin had no effect on the blood pressure of D5+/+ mice (Fig. 1B). These results suggested a role for NADPH oxidase in the hypertensive response.

Expression of NADPH oxidase proteins and enzyme activity in D5 mice. Expression of gp91phox was investigated using monoclonal antibody 54.1, which recognizes a defined region of gp91phox (4) and is distinct from murine Nox 4. It specifically recognizes a 91-kDa glycoprotein in human cells and a smaller protein of 60–70 kDa in murine cells (4). The difference in size is due to the lower level of glycosylation present in murine gp91phox, which is due to the absence of glycosylation sites (39). Using this antibody, we specifically detected a 65-kDa protein in mouse kidney and brain, corresponding to murine gp91phox (Fig. 2). The expression of gp91phox was higher in the kidney and brain of D5−/− than D5+/+ mice on a normal salt diet. In addition, renal Nox 4 expression was greater in D5−/− than in D5+/+ mice (Fig. 2). Cytosolic subunit p47phox expression was also higher in the kidney and brain of D5−/− than D5+/+ mice on a normal salt diet.
In Vitro Studies

Either the D5 receptor (indicated by increased systemic oxidative stress in D5 mice) or the D5 +/+ mice on a high-salt diet (Fig. 3A) showed increased NADPH oxidase activity in kidney membranes from D5+/+ and D5+/+ mice on a normal (0.8%) NaCl diet. Results are expressed as light units per microgram protein per minute (n = 3–6; *P < 0.05 vs. D5+/+ mice; #P < 0.05 vs. others in the D5+/− group, ANOVA, Scheffe’s test).

Plasma TBARS. Plasma TBARS levels were higher in D5+/− than in D5+/+ mice on a chronic sodium load, indicating increased systemic oxidative stress in D5+/− mice. Apocynin decreased TBARS levels (consistent with the reduction in NADPH oxidase activity) in D5+/− mice but not in D5+/+ mice (Fig. 4).

In Vitro Studies

Because of the absence of the D5 in vivo results in an increase in expression of NADPH oxidase and an increase in ROS production, we surmised that the D5 activation may act to decrease the production of ROS by inhibiting the expression of NADPH oxidase subunits, specifically gp91phox, p47phox, and Nox 4. To show that the D3 stimulation directly inhibits the production of ROS, studies were performed in HEK-hD5 cells. Radioligand binding assay [3H-SCH23390 with specific binding defined by 1 μM (+)-butaclamol] in this cell line showed a maximum receptor density of 2,400 fmol/mg protein and a dissociation constant of 0.80 nM (59). Basal cAMP accumulation was greater in hD5-transfected HEK-293 cells than in empty vector-transfected HEK-293 cells, indicating constitutive activity, in agreement with previous reports (48). cAMP production in hD5 cDNA-expressing cells was significantly increased by D3 agonist fenoldopam, and SQ22536, an adenylyl cyclase inhibitor (43), inhibited fenoldopam-stimulated cAMP production in HEK-hD5 cells (data not shown).

Expression of NADPH oxidase proteins and enzyme activity in HEK-hD5 cells. In HEK-hD5 cells, fenoldopam decreased gp91phox expression in a dose- and time-dependent manner (Fig. 5, A and B). At least 10−8 mol/l fenoldopam was needed to inhibit gp91phox expression and ~6 h was needed for the inhibitory effect to become evident. No effect was seen in nontransfected and empty vector-transfected HEK-293 cells (data not shown). gp91phox expression was not different between nontransfected and empty vector-transfected HEK-293 cells from HEK-HD5 cells (data not shown), suggesting the constitutive action of D3 on cAMP production did not translate to constitutive inhibition of gp91phox expression. In HEK-HD5 cells, the D2-like receptor antagonist, SCH23390, by itself had no effect on gp91phox expression, indicating that it had no constitutive inhibitory effect on gp91phox protein expression. However, SCH23390 treatment did prevent the inhibition of gp91phox expression by fenoldopam in the cell line (Fig. 5C). Because the D3 but not D2 is heterologously expressed in these cells, SCH23390 is essentially a D3 antagonist, and therefore the inhibitory action of fenoldopam is exerted at the D3 receptor.

A 30-min incubation of HEK-hD5 cells with fenoldopam (1 μmol/l), induced a decrease in NADPH-dependent chemiluminescence.
nescence in the membrane preparations. The D1-like antagonist SCH23390 by itself did not affect NADPH oxidase activity. However, the inhibition of NADPH oxidase activity was blocked by SCH23390 (1 μmol/l), indicating action at the D5, but not by the PKA inhibitor H-89 (1 μmol/l), indicating that the effect was independent of PKA. The specificity of the enzymatic reaction was confirmed using DPI, which completely abolished NADPH-dependent O₂⁻ production (Fig. 6).

We next examined the effect of D₅ activation on the regulation of O₂⁻ production. Fenoldopam inhibited O₂⁻ production in a dose- and time-dependent manner (Fig. 7, A and B). The inhibition of O₂⁻ production occurred sooner than the inhibition of gp91phox expression, indicating that D₅-mediated inhibition of NADPH activity occurs via a mechanism other than a decrease in oxidase protein expression. The specificity of fenoldopam as a D₅ agonist in these cells was supported by studies with SCH23390, which had no effect by itself, indicating the absence of a constitutive inhibition of O₂⁻ production by D₅ signaling. The inhibitory effect of fenoldopam on O₂⁻ production was not prevented by an adenyl cyclase inhibitor (SQ22536) or PKA inhibitors (H-89, Rp-8-piteridino-cAMPS, and Rp-cAMPS) (Fig. 7C), whereas a similar concentration of SQ22536 prevented the stimulatory effect of fenoldopam on cAMP production in HEK-hD₅ cells (data not shown). We used concentrations of PKA inhibitors that have been reported to inhibit PKA activity in HEK-293 cells (24, 38, 46). Thus these data and those shown in Fig. 6 strongly suggest that the inhibitory effect of fenoldopam on O₂⁻ production was independent of cAMP/PKA.

Previously, we reported that hD₅ stimulation inhibits PLD2 expression and activity (59). Because the product of PLD2 activity, phosphatidic acid, activates NADPH oxidase and because PLD can be activated independently of cAMP (31, 53, 59), we next investigated the consequence of hD₅ activation on O₂⁻ production in the absence of PLD2 (Fig. 8). The dominant negative PLD2, K758R-PLD2 (44, 59), expressed in HEK-hD₅ cells decreased basal O₂⁻ production and attenuated, but did not prevent, the inhibitory effect of fenoldopam on O₂⁻ production, indicating that inhibition of PLD2 mediated by hD₅ stimulation was only partially responsible for the inhibitory effect on NADPH activity. The dominant negative PLD2 had no effect on gp91phox expression (data not shown).

We also quantified H₂O₂ production in HEK-hD₅ cells by the homovanillic acid oxidation method (54). Consistent with
the inhibitory effect of fenoldopam on $O_2^-$ production, this D₁-like receptor agonist also inhibited $H_2O_2$ production in a dose- and time-dependent manner (Fig. 9, A and B).

**DISCUSSION**

In previous studies, we reported that disruption of the D₅ in mice resulted in increased blood pressure and that increased blood pressure in anesthetized D₅⁻/⁻ mice was acutely normalized by inhibition of the V₁ vasopressin, oxytocin, glutamate, and α-adrenergic receptors (27). We now report that increased generation of ROS may be responsible for the high blood pressure in D₅⁻/⁻ mice. Consistent with this idea, we show increased expression of NADPH oxidase proteins (gp91phox and p47phox) and NADPH oxidase activity in brain and kidney of D₅⁻/⁻ mice relative to D₅⁺/+ littermates. Similarly, renal Nox 4 expression was also increased in D₅⁻/⁻ mice. Plasma TBARS, an index of systemic oxidative stress, was increased in D₅⁻/⁻ mice, indicating enhanced ROS production in these animals. Furthermore, chronic administration of apocynin, a drug with antioxidant activity, normalized blood pressure, although it did not decrease the expression of gp91phox and p47phox in D₅⁻/⁻ mice. This was not unexpected because apocynin decreases NADPH oxidase activity by preventing assembly of the NADPH oxidase subunits rather than altering protein expression. Nevertheless, apocynin normalized NADPH oxidase activity in brain and kidney and plasma TBARs in D₅⁻/⁻ mice, suggesting that the D₅ activation may promote normal blood pressure by preventing excessive ROS production.

Dopamine has contrasting, concentration-dependent effects on ROS production, acting as an antioxidant at physiologically relevant concentrations, but as a prooxidant at high concentrations. Indeed, high concentrations of dopamine or D₁-like receptor agonist SKF38393, can increase the generation of $O_2^-$ and $H_2O_2$ (11). ROS can be produced by dopamine transporter uptake-dependent and dopamine receptor-independent mechanisms (2), and excessive stimulation of D₂ receptors can also
increase ROS production (58). However, the protective effects of apomorphine on oxidative stress-induced cell death are, at least in part, mediated by dopamine D4 receptors via the regulation of cGMP-operated Ca2+ channels (30). D4 receptor activation has also been associated with antioxidant effects (8). We show here that activation of the D5 inhibited expression of gp91phox, suggesting the possibility that increased NADPH oxidase activity and ROS production in D5−/− mice were caused by an increased expression of NADPH oxidase subunits. However, the acute inhibition of NADPH oxidase activity and the generation of both O2•− and H2O2 by the D5 activation cannot be adequately explained by inhibition of gp91phox expression.

D1-like receptor activation has been shown to induce antioxidant effects via PKA (60), and activation of both the D1 and D5 increases cAMP accumulation and PKA activity. Additionally, cAMP/PKA is involved in the inhibition of superoxidation in mesangial cells and neutrophils (12). In our study, however, neither the inhibition of NADPH oxidase activity nor the decreased generation of ROS by D5 signaling was prevented by adenylyl cyclase or PKA inhibitors, indicating that this pathway was also not essential in D5/NADPH-mediated inhibition in ROS production.

Activation of D1-like receptors has been shown to decrease oxidative stress in renal tubular (59), vascular smooth muscle (60), and brain cortical cells (37). Activation of both members of the D1-like receptor family, D1 and D5, has been suggested to mediate antioxidant effects. For example, Yasunari and colleagues (60) reported that inhibition of expression of either D1 or D5 by antisense oligonucleotides in renal vascular smooth muscle cells impaired antioxidant properties. Because antisense oligonucleotides cannot completely silence the gene of interest, we studied the antioxidant responses mediated by D5 (independent of D1) heterologously expressed in HEK-293 cells. Previously, we reported that D5 activation resulted in inhibition of PLD2 activity (59). In the present study, we found that the inhibitory effect of fenoldopam on O2•− production was decreased but not abolished in HEK-hD5 cells expressing catalytically inactive PLD2 (44), indicating PLD2 is only partially involved in the D5 regulation of NADPH oxidase activity.

The non-PKA/non-PLD mechanism by which the D5 acutely decreased NADPH oxidase activity is still unclear. In preliminary studies, we found in HEK-hD5 cells that D5 colocalized with gp91phox in cell surface membranes and that D5 stimulation induced the dissociation of D5 and gp91phox and impaired the translocation of p67phox, a cytosolic component of the NADPH oxidase component, to the oxidase complex (34). More recently, we have found that stimulation of D5 in HEK-hD5 cells increased heme oxygenase-1 activity (unpublished observations). Heme-oxygenase-1 converts heme to carbon monoxide, iron, and biliverdin (36). Carbon monoxide can inhibit NADPH oxidase activity (61) and bilirubin, a product of the action of biliverdin reductase on biliverdin, also has antioxidant activity toward O2•− (42). Thus D5 activation may lead to inhibition of ROS production indirectly by this mechanism. However, PKA has been reported to be involved in the activation of heme oxygenase-1 (29). We, therefore, favor the hypothesis that the D5 interferes with the distribution and assembly of NADPH oxidase components, causing the inhibition of NADPH oxidase activity.

In the present study, we were not able to determine directly whether the increased generation of ROS in D5−/− mice was a cause or a consequence of hypertension. However, a modest increase in sodium intake (4% vs. a normal salt diet of 0.8% NaCl) elevated blood pressure further in the D5−/− mice on a C57BL/6 background but had no effect on renal NADPH oxidase expression or activity. Moreover, in vitro studies showed that NADPH oxidase activity and the expression of NADPH oxidase subunits can be decreased by activation of the D5. Therefore, we suggest that the high blood pressure in D5−/− mice resulted from increased ROS production, resulting from enhanced NADPH oxidase protein expression and enzymatic activity. However, high-salt intake increased blood pressure further in D5−/− mice, without further increasing renal NADPH oxidase activity, indicating that the increase in blood pressure caused by high salt intake cannot be entirely explained by increased production of ROS in the kidney.

In summary, we have found that stimulation of the D5 results in an antioxidant response that is mediated by inhibition of NADPH oxidase activity, in the short term, and inhibition of NADPH oxidase expression, in the long term. D5−/− mice are hypertensive, salt sensitive, and have increased systemic oxi-
The constitutive D5-mediated increase in cAMP accumulation regulates ROS levels. However, the current studies show that associated with a higher basal superoxide levels compared with has a decreased ability to stimulate cAMP production, is mediated by increased ROS production because apocynin, does not explain D5-mediated inhibition of ROS production. It therefore, the D5 may provide an ongoing surveillance to perspectives

The D5, unlike the D1, is constitutively activated (48); therefore, the D5 may provide an ongoing surveillance to regulate ROS levels. However, the current studies show that the constitutive D5-mediated increase in cAMP accumulation does not explain D5-mediated inhibition of ROS production. It is still possible, however, that activation of the D5 may lead to inhibition of ROS production when ROS activity is increased. In Chinese hamster ovary cells, a D5 (F173L) variant, which has a decreased ability to stimulate cAMP production, is associated with a higher basal superoxide levels compared with cells expressing wild-type D5. Conversely, a D5 (S390G) with increased ability to stimulate cAMP production is associated with lower basal superoxide levels compared with the wild-type D5 (unpublished observations).

GRANTS

These studies were supported, in part, by the National Institutes of Health through Grants HL-23081, DK-39308, HL-68686, DK-52612, HL-074940, and AR-042426.

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