Phosphatidylinositol 3-Kinase-dependent Suppression of the Human Inducible Nitric-oxide Synthase Promoter Is Mediated by FKHRL1*§

The synthesis of nitric oxide by inducible nitric-oxide synthase (iNOS) plays an important role in the innate immune response by promoting microbial killing and cell damage. In response to inflammatory cytokines and bacterial products, the human iNOS (hiNOS) gene undergoes rapid transcriptional activation via binding of stimulatory transcription factors (e.g. AP-1 and NF-κB) to its 5′-flanking region. However, maximal hiNOS promoter induction was suppressed via an unknown phosphatidylinositol 3-kinase (PI3K)-dependent mechanism. We hypothesized that inhibition of the transcription factor FKHRL1 by the PI3K/protein kinase B pathway attenuates hiNOS promoter induction by bacterial lipopolysaccharide and interferon-gamma (LPS/IFN-γ). Human lung epithelial adenocarcinoma (A549) cells were transiently transfected with an 8.3-kb hiNOS promoter luciferase reporter construct. Co-expression of dominant-negative protein kinase B potentiated LPS/IFN-γ-stimulated hiNOS promoter activity. In response to LPS/IFN-γ, FKHRL1 was phosphorylated in a PI3K- and time-dependent fashion. Co-expression of constitutively active FKHRL1 increased hiNOS promoter activity and mRNA levels. Dominant-negative siRNA expression showed that FKHRL1 was necessary for the inhibitory effects of PI3K on hiNOS induction. The same effect was observed upon mutation of a consensus FKHRL1-binding site in the hiNOS promoter. By gel-shift analysis, the corresponding oligonucleotide probe bound endogenous FKHRL1 in an LPS/IFN-γ- and PI3K-sensitive fashion. Regulation of the hiNOS promoter by FKHRL1 represents a potentially important molecular mechanism by which the PI3K pathway might suppress pro-inflammatory and pro-apoptotic responses to cytokines and bacterial products.

NO is an important signaling molecule produced by a family of nitric-oxide synthases (NOS)2 during the conversion of L-arginine to L-citrulline (1). Both constitutive and inducible NOS isoforms have been described (2). Biological processes regulated by calcium-dependent constitutive NO synthesis include vascular tone and neurotransmission and are catalyzed by the neuronal and endothelial NOS isoforms, respectively (3–5). In contrast, induction of the calcium-independent inducible isoform of NO synthase (iNOS, or NOS2) by inflammatory stimuli results in the sustained release of higher levels of NO (1, 6). In vitro and in vivo studies demonstrated a role for iNOS-derived NO in tissue injury, likely via oxidative DNA and membrane damage, inhibition of cellular respiration, and direct modification of proteins (7). Moreover, depending on cell type or stimulus, NO production was directly linked to the initiation of, or protection from, cellular apoptosis (8).

Activation of human iNOS (hiNOS) transcription is central to the cellular response to inflammatory stimuli. Cytokine-activated signaling proteins are involved in iNOS gene expression and de novo NO production (e.g. mitogen-activated protein kinases (MAPKs), Janus tyrosine kinase/signal transducer and activator of transcription (STAT), protein kinase A, and protein kinase C) (9–11). Studies of the hiNOS 5′-flanking region identified the involvement of several inducible transcription factors. The hiNOS promoter contains enhancer regions located up to 16 kb upstream of the transcription start site (12). Two AP-1 and two NF-κB binding sites were necessary for cytokine stimulation of hiNOS promoter activation (13). Specific AP-1 heterodimers (JunD/Fra-2) bound the hiNOS promoter in extracellular signal-regulated kinase- and p38 MAPK-dependent fashion, providing one molecular mechanism for hiNOS transcriptional activation (14). In separate studies, NF-κB or c-Fos interacted with STAT-1 to regulate hiNOS promoter activity in response to cytokines (10, 15).

Because overproduction of NO by iNOS might cause abnormal inflammation, cell proliferation, or apoptosis, signaling mechanisms that suppress basal iNOS promoter activity have

* This work was supported by the Division of Intramural Research, NHLBI, National Institutes of Health (to J. M., A. S. K., and S. N.) and a Canadian Institutes for Health Research Operating Grant (to A. S. K., J. F., and A. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

‡ To whom correspondence should be addressed: McGill University, Royal Victoria Hospital, 687 Pine Ave. W., Rm. L3.05, Montreal, Quebec H3A 1A1, Canada. Tel.: 514-843-1664; Fax: 514-843-1686; E-mail: arnold.kristof@muhc.mcgill.ca.

§ The abbreviations used are: NOS, nitric-oxide synthase; iNOS, inducible NOS; hiNOS, human iNOS; FKHRL1, forkhead (Drosophila) homolog (rhabdomyosarcoma)-like 1; LPS, lipopolysaccharide; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; SEAP, soluble excreted alkaline phosphatase; IFN, interferon; HA, hemagglutinin; dn, dominant negative; GST, glutathione S-transferase.

2 The abbreviations used are: NOS, nitric-oxide synthase; iNOS, inducible NOS; hiNOS, human iNOS; FKHRL1, forkhead (Drosophila) homolog (rhabdomyosarcoma)-like 1; LPS, lipopolysaccharide; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; SEAP, soluble excreted alkaline phosphatase; IFN, interferon; HA, hemagglutinin; dn, dominant negative; GST, glutathione S-transferase.
attracted growing interest. In contrast to MAPKs and Janus tyrosine kinases, phosphatidylinositol 3-kinase (PI3K) activity limited hiNOS gene induction (16). PI3K is a key signaling enzyme in cellular responses to growth factors and cytokines (i.e. transcriptional activation, initiation of protein translation, cell proliferation, vesicular trafficking, cytokinesis) (17, 18). Binding of the PI3K p85 subunit to activated receptors leads to recruitment of the p110 catalytic subunit to membrane compartments (19). The resulting phosphorylation of phosphatidylinositol moieties initiates pleckstrin homology domain-dependent translocation of proteins to membranes, and activation of downstream kinases (e.g. phosphoinositide-dependent kinase and protein kinase B (PKB)) (20). Via this mechanism, PI3K can stimulate the activity of transcription factors such as STAT1, AP-1, and NF-κB (21–23). However, PI3K suppressed hiNOS transcription (16, 24, 25), and other inhibitory mechanisms are likely to play a role.

The forkhead transcription factors AFX, FKHR, and FKHRL1 are phosphorylated by PKB (26). Also known as FOXO3A, FKHRL1 activates the promoters of genes involved in cell-cycle arrest or apoptosis (e.g. p27kip1, IGFBP-1, Bim, and TRAIL) (27–30). Phosphorylation of FKHRL1 at Thr-32 or Ser-253 led to a reduction in nuclear FKHRL1, resulting in the attenuation of FasL-dependent apoptosis (31). Several consensus binding sites for FKHRL1 are present in the hiNOS 5'-flanking region. We hypothesized that, in the presence of inflammatory mediators, the PI3K pathway can dampen hiNOS promoter induction via PKB-dependent phosphorylation and suppression of FKHRL1. Here, we show that PI3K inhibits hiNOS promoter activity in a PKB-dependent fashion. Moreover, LPS stimulates FKHRL1 phosphorylation, and FKHRL1 regulates the hiNOS promoter via a specific enhancer sequence. These data establish a role for FKHRL1 in the attenuation of LPS-dependent hiNOS gene transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cytokine Induction**—A549 cells (American Type Culture Collection (ATCC) CCL 185), a human lung alveolar type II epithelial cell adenocarcinoma cell line, were grown as previously described (14). To initiate experiments, cells were washed with serum-free medium and treated with or without inhibitors for 1 h before incubation with LPS, 0–100 μg/ml (Sigma), without or with IFN-γ, 100 units/ml. Wortmannin (Biomol) dissolved in Me2SO was added directly to the cells as indicated.

Generation of hiNOS Promoter Mutants—Potential FKHRL1-binding sites (consensus sequence 5'-RTAAAYA-3', where R = C or T, and Y = A or G) were identified in the wild-type 8.3-kb hiNOS promoter fragment (GenBankTM accession number AF017634). The 8.3-kb hiNOS promoter fragment linked to the firefly luciferase cDNA (PGL3-hiNOS) was previously described (32). Mutations in putative FKHRL1-binding sequences were generated by site-directed mutagenesis (Stratagene). The positive strands of the double-stranded oligonucleotides corresponding to the identical regions of the hiNOS promoter were as follows (mutated (mt) sequences are underlined, and nomenclature used in the text is indicated in boldface): wt −749 (−747 to −741), 5'-ATAAATATATTATAA-3'; mt −749, 5'-ATCACTGACCTCCATA-3'; wt −1532 (−1532 to −1526), 5'-TGTTTAC-3'; mt 1532, 5'-TTGTACCC-3'; wt −2766 (−2766 to −2760), 5'-TGTTTAC-3'; mt −2766, 5'-TGTTTAC-3'; wt −2901 (−2901 to −2895), 5'-TATACCA-3'; and mt −2901, 5'-TATACCA-3'. Mutations were confirmed by automated sequencing (ABI Prism 3100, Applied Biosciences).

**Transient Transfection and Determination of Promoter Activity**—A549 cells were transfected with thymidine kinase promoter construct linked to Renilla luciferase cDNA (pRL-TK, 25 ng/ml), and wild-type or mutant PGL3-hiNOS, 1 μg/ml (32), without or with mammalian expression vectors containing the cDNA encoding dominant-negative PKBα (pUSE-amp-PKB-dn, 1 μg/ml, Upstate Biotechnology) and in separate experiments with wild-type or constitutively active HA-tagged FKHRL1 (1 μg/ml, gift from Drs. M. Greenberg and A. Brunet, Harvard University) (31). A pECE mammalian expression vector containing cDNA for HA-tagged dominant-negative FKHRL1 lacking the transactivation domain (0.5 μg/ml, dn FKHRL1, base pairs 1–1789), or psiRNA expression vectors (Invitrogen) containing cDNA-encoding anti-FKHRL1 siRNA (0.5 μg/ml, aFKHRL1 siRNA, target sequence 5'-GGATAAGGCGCAGCAACA-3') or scrambled control siRNA, were used to inhibit endogenous FKHRL1 protein levels or activity. Experiments were also performed using NF-κB or pAP-1 reporter vectors (1 μg/ml, pNF-κB-SEAP or pAP-1-SEAP, Clontech) transfected with 8 μl of Lipofectamine, and SEAP activity was normalized to cell lysate total protein concentrations. After 36 h, cells were washed with serum-free medium, and incubated without or with wortmannin before the addition of LPS/IFN-γ for 6 h, and harvesting for determination of luciferase activity (Dual luciferase kit, Promega, or Great.
FKHRL1 Regulates the Human iNOS Promoter

**A**

| NF-κBu | Wort | Cold WT | Cold MT |
|--------|------|---------|---------|
| 7±3    | -    | +       | +       |
| 21±5   | -    | +       | +       |
| 214±13 | -    | +       | +       |
| 149±2  | -    | +       | +       |
| 32±10  | -    | +       | +       |
| 127±15 | -    | +       | +       |

| NF-κBd | Wort | Cold WT | Cold MT |
|--------|------|---------|---------|
| 112±7  | -    | +       | +       |
| 87±8   | -    | +       | +       |
| 162±18 | -    | +       | +       |
| 142±5  | -    | +       | +       |
| 70±3   | -    | +       | +       |
| 164±2  | -    | +       | +       |

| AP-1u  | Wort | Cold WT | Cold MT |
|--------|------|---------|---------|
| 104±7  | -    | +       | +       |
| 68±16  | -    | +       | +       |
| 161±18 | -    | +       | +       |
| 127±12 | -    | +       | +       |
| 39±24  | -    | +       | +       |
| 170±7  | -    | +       | +       |

| AP-1d  | Wort | Cold WT | Cold MT |
|--------|------|---------|---------|
| 62±7   | -    | +       | +       |
| 36±3   | -    | +       | +       |
| 140±3  | -    | +       | +       |
| 119±3  | -    | +       | +       |
| 27±23  | -    | +       | +       |
| 105±37 | -    | +       | +       |

**B**

**FIGURE 2.** The effect of wortmannin on LPS/IFN-γ-stimulated NF-κB and AP-1 activity and binding to regulatory sites in the hiNOS promoter. A, A549 cells were incubated for 1 h without or with 200 nM wortmannin and LPS/IFN-γ for 3 h before preparation of nuclear lysates. Samples (5 μg) of nuclear protein were incubated with 32P-labeled oligonucleotides encoding specific upstream and downstream NF-κB and AP-1 binding sequences in the hiNOS promoter. Specificity of binding reactions was tested by adding a 100-fold excess of unlabeled wild-type (WT) or mutated (MT) oligonucleotide 15 min before the labeled probe to samples from cells exposed to agonist without inhibitor. Autoradiographs are representative of four experiments. The mean band density (±S.E.), as calculated for each experimental condition from four separate experiments, is recorded below each gel lane (full gels in supplemental Fig. S1). B, A549 cells transiently transfected with soluble excreted alkaline phosphatase (SEAP) reporter plasmids containing NF-κB or AP-1 consensus-binding sites were incubated for 1 h with serum-free medium or 200 nM wortmannin, then without or with LPS/IFN-γ for 6 h before assay of culture medium for SEAP activity, and cell lysis for protein determination. Data are means of values from one experiment with assays in triplicate (±S.E.) and are representative of two to three experiments. Statistical significance was established in each experiment. *p < 0.05 by Student’s t test for the indicated comparisons. NS, not significant.

**Measurement of hiNOS mRNA Levels**—A549 cells were lysed in TRizol reagent (Invitrogen) before chloroform extraction and precipitation of RNA as per the manufacturer’s protocol. RNA (2 μg) was reverse transcribed (Superscript II, Invitrogen), and 1.5 μl of cDNA template was combined with 1 μl of carboxyfluorescein (FAM)-labeled and verified TaqMan probe for 18 S rRNA or hiNOS (Applied Biosystems, assay numbers Hs99999901_s1 or Hs00167257_m1, respectively) in 1× TaqMan universal PCR master mix (Applied Biosystems), for a total of 20 μl. Real-time PCR was performed under the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 55 °C for 1 min (7500 Real Time PCR System, Applied Biosystems). Gene expression relative to the experimental control was calculated using the ΔΔCt method (33). Ct values for hiNOS mRNA or 18S RNA were estimated from triplicate samples and averaged. ΔCt values were derived by subtracting average 18S Ct from average hiNOS Cτ. ΔΔCt values were derived by subtracting control ΔCt values from test ΔCt values. Fold increase in mRNA levels relative to untreated empty vector control = 2− ΔΔCt.

**Measurement of FKHRL1 Protein Levels and Phosphorylation**—A549 cells were incubated without or with wortmannin for 1 h, and then LPS without or with IFN-γ as indicated, before lysis in homogenization buffer (20 mM Tris, pH 8.0, 1% Nonidet P-40, 1 mM EDTA, 5 mM benzamidine, aprotinin, 10 μg/ml, leupeptin, 10 μg/ml, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 100 μM sodium orthovanadate). After freezing and thawing, lysates were centrifuged for 5 min at 1,000 × g before collection of the supernatants and centrifugation for 30 min at 14,000 × g. For each experiment, equal amounts of total protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection of total FKHRL1 or FKHRL1 phosphorylated at Ser-253 was performed by Western blot analysis using anti-FKHRL1 or anti-phospho-FKHRL1 Ser-253 antibodies (Santa Cruz Biotechnology).

**Oligonucleotides Used in Electrophoretic Mobility Gel Shift Assay**—Oligonucleotides were synthesized by Invitrogen. The positive strands of the double-stranded oligonucleotides corresponding to the identical regions of the hiNOS or IGFBP-1 promoter were as follows (mutated sequences are underlined): wt NF-κBu (−8287 to −8270), 5′-CCCTGGGGAACTCCT-
GCA-3'; mt NF-κBu, 5'-CCCTATAGAATACTGCA-3'; wt NF-κBd (−102 to −119), 5'-GCTGGGGGACACTCCCTT-3'; mt NF-κBd, 5'-GCTGATACACTAACTTT-3'; wt AP-1u (−5307 to −5290), 5'-CCAGCTTAGTGTCACACTC-3'; mt AP-1u, 5'-CCAGCTTAAATTACACTC-3'; wt AP-1d (−5121 to −5104), 5'-TTGTGTAGCTACGCCCC-3'; mt AP-1d, 5'-TTTTGTTGTAATACCCCC-3'; −1532 (−1540 to −1520); 5'-CTGAGTGCCTGTACCTGACC-3'; and IRS, 5'-ATTGCTAGCAAAACAAACCGTACGTTA-3' (31). Complementary strands in equal concentrations were mixed and annealed by slowly cooling to room temperature after heating to 95 °C for 5 min. For electrophoretic mobility shift assay, oligonucleotide probes (10 pmol) were labeled with [γ-32P]ATP using a T4 polynucleotide kinase kit and purified on G-25 spin columns (all from Amersham Biosciences).

Preparation of Nuclear Extracts and Electrophoretic Mobility Gel Shift Assay—A549 cells grown to near confluence were incubated for 1 h without or with wortmannin, before stimulation with LPS/IFN-γ for 4 h, and isolation of nuclear proteins as previously described (13, 34). Samples (5 μg) of nuclear proteins were incubated with a radiolabeled probe containing hiNOS promoter AP-1, NF-κB, or FKHRL1 binding sequences, or one containing the IRS sequence in the IGF/IGFBP-1 promoter (31), for 20 min at room temperature (−21 °C). Specificities of the binding reactions were tested in competition assays in which a 10- or 50-fold excess of unlabeled wild-type or mutant oligonucleotide was added to extracts from stimulated cells 15 min before the labeled probe. Protein-nucleotide complexes were separated by electrophoresis in a 6% DNA-retardation gel (Invitrogen) with Tris borate-EDTA (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.3) at constant current (30 mA) at room temperature. Photographic film was exposed to dried gels at −70 °C and scanned using an Epson Expression 636 scanner.

RESULTS

PI3K and PKB Mediate LPS/IFN-γ-dependent Suppression of hiNOS Promoter Activity in NF-κB- and AP-1-independent Fashion—Previous studies implicated the PI3K pathway as a negative regulator of LPS-dependent iNOS and NO synthesis (16, 24, 35, 36). In murine macrophages, the PI3K inhibitor wortmannin potentiated LPS activation of the iNOS promoter (16). Similar experiments using wortmannin in human lung epithelial adenocarcinoma (A549) cells demonstrated an inhibitory role for PI3K in human iNOS promoter induction (25). PKB can mediate PI3K effects on gene transcription (37). Like wortmannin, overexpression of dominant-negative PKB enhanced LPS/IFN-γ-stimulated hiNOS promoter activity (Fig. 1). Co-expression of dominant-negative PKB also increased basal promoter activity. The -fold stimulation of hiNOS promoter activity by LPS/IFN-γ (LPS/IFN-γ-stimulated divided by basal hiNOS promoter activity = 1) was 3.6 in empty vector-transfected cells and 5.6 in dominant-negative PKB-transfected cells. These results suggest that a PI3K- and PKB-dependent transcription factor might regulate basal hiNOS transcription and mediate a dampening effect during exposure to innate immune mediators.

STAT1 is necessary for hiNOS transcriptional induction; however, our study in A549 cells failed to demonstrate an effect of wortmannin on activation of STAT1 (25). Two NF-κBu (NF-κBu, −8283 bp; NF-κBd, −115 bp) and two AP-1 (AP-1u, −5301 bp; AP-1d, −5115 bp) binding sites in the hiNOS 5'-flanking region are necessary for full hiNOS transcriptional induction by cytokines and LPS (13, 14). We determined whether wortmannin-dependent increases in NF-κB or AP-1 binding or activity might explain enhanced hiNOS promoter activity. By gel-shift analysis, wortmannin reduced LPS/IFN-γ-stimulated binding to the AP-1d site (Fig. 2A) but had little effect on AP-1 activity (Fig. 2B), indicating that the potentiation of hiNOS promoter activity by wortmannin could not be explained by effects on AP-1. Moreover, wortmannin did not significantly alter LPS/IFN-γ-stimulated NF-κB binding or reporter activity (Fig. 2, A and B). These data support AP-1- and NF-κB-independent transcriptional regulation of hiNOS by the PI3K pathway.

FKHRL1 Regulates the Human iNOS Promoter

A  p-FK  total FK
B  p-FK  total FK
L/I (hr)    0.5  1  2  4
−−+−+−
−−+−+−

C  p-FK  total FK
L/I Wort LPS IFN-γ
−−−−−−
−−−−−−
−−−−−−

D  p-FK  total FK
LPS (μg/ml)  −1  10  100
−−−−−−
−−−−−−
−−−−−−

FIGURE 3. The effect of LPS and IFN-γ on PI3K-dependent phosphorylation of FKHRL1. A549 cells were incubated without or with 200 nm wortmannin for 1 h in serum-free medium, and then LPS/IFN-γ (L/I) for the indicated durations (A), without or with LPS/IFN-γ in the absence or presence of 200 nM wortmannin for 1 h (B), LPS, IFN-γ, or both for 1 h (C), or the indicated concentrations of LPS for 1 h (D). Equal amounts of proteins from whole cell lysates were separated by SDS-PAGE, and immunodetection of phospho-FKHRL1 (p-FK) or FKHRL1 (total FK) was performed by Western blot analysis. Data are representative of three experiments.
FKHRL1 Regulates the Human iNOS Promoter

FKHRL1 binding sites (rtaaaya)

FIGURE 4. FKHRL1 regulates activation of hiNOS transcription. A, putative FKHRL1-binding sites in the hiNOS 5' flanking region based on the FKHRL1 consensus binding sequence 5'–RTAAAAYA–3'. B, A549 cells transiently transfected with PGL3-hiNOS plus vector alone (EV), that overexpressing wild-type FKHRL1 (WT), or that overexpressing constitutively active FKHRL1 (TM), were incubated for 1 h without or with 200 nm wortmannin, and then for 6 h without or with LPS/IFN-γ. hiNOS promoter activity is the firefly luciferase divided by renilla luciferase activity = 1. Data are means of values from one experiment with assays in triplicate (± S.E.) and are representative of four experiments. Statistical significance was established in each experiment. p = 0.0001 by three-way analysis of variance; by post-hoc testing, there was an independent effect of LPS/IFN-γ, wortmannin, and transfected vector, respectively. There was a statistically significant difference attributed to wortmannin in empty vector— but not in wild-type- or triple mutant-overexpressing cells. C, A549 cells transiently transfected with vector alone (EV), or that overexpressing constitutively active FKHRL1 (TM), were incubated for 1 h with serum-free medium, and then for 6 h without or with LPS/IFN-γ. Real-time PCR was performed on reverse-transcribed RNA as detailed under "Experimental Procedures." Data are the mean -fold increases in hiNOS mRNA copy number compared with empty vector control and using 18 S RNA levels as the endogenous calibrator (ΔΔCt method). Results represent the mean of three experiments with assays in triplicate (± S.E.). *, p < 0.05 by Student's t test.

LPS Enhances the Phosphorylation of FKHRL1 in a PI3K-dependent Fashion—FKHRL1, a DNA-binding activator of gene transcription, can be phosphorylated and inhibited by PKB. In a previous study, a combination of LPS and IFN-γ led to maximal hiNOS promoter induction, whereas each alone had little effect (14). To better define the contributions of individual signaling pathways, we assessed the phosphorylation status of FKHRL1 in whole cell lysates from A549 cells incubated with LPS and/or IFN-γ. In the presence of LPS/IFN-γ, phosphorylation of FKHRL1 at Ser-253 increased within 30 min, and persisted unchanged for at least 4 h, whereas total FKHRL1 levels remained constant (Fig. 3A). In separate experiments, serum-starved A549 cells were incubated without or with LPS/IFN-γ for 1 h. Consistent with PI3K regulation of FKHRL1, wortmannin inhibited basal and LPS/IFN-γ-stimulated phosphorylation of FKHRL1 at Ser-253, with no effect on total FKHRL1 levels (Fig. 3B).

We next assessed the relative role of LPS or IFN-γ in the phosphorylation of FKHRL1. Administration of LPS alone led to phosphorylation of FKHRL1 at Ser-253, whereas IFN-γ had little effect (Fig. 3C). Phosphorylation of FKHRL1 in response to LPS occurred independently of IFN-γ. Neither LPS nor IFN-γ alone affected total FKHRL1 levels. The maximal effect occurred at LPS concentrations between 1 and 10 μg/ml (Fig. 3D). Thus, FKHRL1 is a downstream target of wortmannin-sensitive signal transduction in response to LPS.

FKHRL1 Is a Cis-activator of the hiNOS Promoter—Based on its consensus DNA-binding sequence (i.e. 5'–RTAAAAYA–3'), several putative FKHRL1-binding sites were present in the hiNOS 5' flanking region (Fig. 4A). Three overlapping sites were present between 747 and 733 bases upstream of the transcription start site (positions –747 to –733, designated –747). Unique consensus binding sites were also present at positions –1532 and –2766 on the antisense strand, and –2901 on the sense strand. To determine whether FKHRL1 can regulate the hiNOS promoter, plasmids expressing wild-type and mutant forms were co-transfected with PGL3-hiNOS. In A549 cells transfected with PGL3-hiNOS and empty vector, those incubated with wortmannin exhibited 3.9-fold induction of hiNOS promoter activity by LPS/IFN-γ, as compared with 2.7-fold in those without (Fig. 4B). Overexpression of wild-type FKHRL1 enhanced basal and LPS/IFN-γ-stimulated hiNOS promoter activity. In cells overexpressing wild-type FKHRL1, induction of hiNOS promoter activity by LPS/IFN-γ was not affected by wortmannin (1.8-fold induction in cells incubated without and with wortmannin, respectively).
These data demonstrate that FKHRL1 is sufficient to increase hiNOS promoter activity.

During overexpression of recombinant wild-type FKHRL1, the influence of PI3K-independent regulators of FKHRL1 (e.g., IκB kinase, serum/glucocorticoid-regulated kinase) could not be ruled out (38, 39). To better isolate the effect of PI3K-dependent inhibition, we overexpressed a mutant form of FKHRL1 that cannot be phosphorylated by PKB. FKHRL1 contains three PKB phosphorylation sites (i.e., Thr-32, Ser-253, and Ser-315). Serine or threonine to alanine mutations at all three sites generated a constitutively active FKHRL1 mutant (see Fig. 4, TM) (31). Overexpression of constitutively active FKHRL1 led to a significant increase in basal and stimulated hiNOS promoter activity that was no longer altered by wortmannin. Additionally, wortmannin did not affect -fold induction of promoter activity by LPS/IFN-γ (one in cells incubated without and with wortmannin, respectively). By real-time PCR, overexpression of constitutively active FKHRL1 increased basal and stimulated hiNOS mRNA levels by 7- and 2-fold versus empty vector control, respectively (Fig. 5B). The -fold induction of hiNOS mRNA by LPS/IFN-γ was 685 in empty vector-transfected cells and 195 in constitutively active FKHRL1-transfected cells. Therefore, in contrast to promoter activity (Fig. 4B), hiNOS mRNA levels remained inducible in cells overexpressing constitutively active FKHRL1 (Fig. 4C), suggesting regulation by additional mechanisms (e.g., mRNA stability). Nonetheless, the results confirm that FKHRL1 can enhance endogenous hiNOS transcription and indicate that FKHRL1-mediated regulation of hiNOS promoter is under inhibitory control by PKB.

FKHRL1 Regulates the Human iNOS Promoter

AUGUST 18, 2006• VOLUME 281 • NUMBER 33
JOURNAL OF BIOLOGICAL CHEMISTRY

23963

FKHRL1 is Required for Enhancement of hiNOS Induction by Wortmannin—To further implicate a dampening role for FKHRL1 in the regulation of hiNOS transcriptional activation, we expressed its dominant-negative mutant or a hairpin siRNA directed against its mRNA. Expression of dominant-negative FKHRL1 and endogenous FKHRL1 knockdown were confirmed by Western blot analysis (see Supplemental Fig. S2). To maximize the probability of detecting the effect of FKHRL1 inhibition, we used a lower concentration of wortmannin (100 nM) but one that was previously shown to potentiate effectively hiNOS promoter induction (25). In A549 cells overexpressing a FKHRL1 mutant lacking its transactivation domain, but not PKB phosphorylation sites, wortmannin no longer potentiated hiNOS mRNA induction (Fig. 5A). In the absence of wortmannin, hiNOS induction was unaffected, indicating that FKHRL1 represents a target for the modulation of hiNOS transcription by PI3K; however, FKHRL1 was not essential for hiNOS gene induction. To rule out the potential contribution of PKB phosphorylation sites to hiNOS induction in the absence of wortmannin, we also used siRNA directed against FKHRL1 mRNA. As seen during overexpression of a FKHRL1 transactivation mutant, knockdown of total FKHRL1 did not affect LPS/IFN-γ

(EV), were incubated for 1 h with serum-free medium, and then for 6 h without or with LPS/IFN-γ. Real-time PCR was performed on reverse-transcribed RNA as detailed under “Experimental Procedures.” Data are the mean -fold increase in hiNOS mRNA copy number compared with empty vector control and using 18 S RNA levels as the endogenous calibrator (ΔΔCt method). Results represent the mean of three experiments each with assays in triplicate (±S.E.). *p < 0.05 by Student’s t test; NS, not significant.
FKHRL1 Regulates the Human iNOS Promoter

induction of hiNOS mRNA but eliminated the ability of wortmannin to enhance hiNOS gene induction (Fig. 5B). Expression of a scrambled siRNA had no effect on the potentiation of hiNOS induction by wortmannin (Fig. 5C). These results indicate that inhibition of FKHR1 transcriptional activity by PI3K-dependent phosphorylation is necessary for PI3K-dependent dampening of hiNOS gene induction.

The hiNOS Promoter Contains a Functional FKHR1-binding Site—To screen for sequences in the hiNOS 5′-flanking region that mediate endogenous FKHR1-dependent effects on promoter activity, PGL3-hiNOS mutants were constructed. Mutation of all three overlapping putative FKHR1-binding sites between −747 and −733 (designated −747) did not significantly affect the ability of wortmannin to enhance LPS/IFN-γ-stimulated promoter activity, nor did mutation of the sequences at −2766 or −2901 (Fig. 6, A and B). The -fold induction values of promoter activity in cells incubated with wortmannin were 6.0, 5.8, 4.8, and 4.8 for the wild-type, −747, −747/−733, and −747/−2901 transfectants, respectively (Fig. 6, A and B). In contrast, mutation of the sequence at −1532 decreased the ability of wortmannin to enhance hiNOS promoter activity in the presence of LPS/IFN-γ, indicating a loss of PI3K-dependent inhibition (Fig. 6, C and D). Also, enhancement of promoter inducibility (−fold induction) by wortmannin was reduced upon mutation of the −1532 site (78- and 42-fold wild-type and −1532 transfectants incubated with wortmannin (Fig. 6C)). Of note, the −1532 mutation also led to a decrease in basal and LPS/IFN-γ-stimulated promoter activity in the absence of wortmannin (Fig. 6C), indicating that not all of its effects could be attributed to wortmannin.

To verify whether FKHR1 can bind its consensus sequence at −1532 bp in the hiNOS promoter (−1532), gel-shift assays were performed using recombinant GST-FKHR1. Purity was assessed by Coomassie staining and Western blot (Fig. 7, A and B). Migration of a 32P-labeled probe containing the −1532 site was retarded by incubation with recombinant GST-FKHR1 but not GST alone (Fig. 7C).

Nuclear proteins were obtained from A549 cells incubated without or with wortmannin, and then without or with LPS/IFN-γ. Regulation of FKHR1 binding was detectable 4 h after incubation with LPS/IFN-γ. After incubation with the −1532 probe, a band was detected at position A (Fig. 7C). Consistent with nuclear FKHR1 binding, the retarded band migrated at the same rate as that corresponding to probe-bound recombinant GST-FKHR1 (band A). Perhaps due to increased molecular weight or altered folding, the GST-FKHR1-DNA complex migrated at a slightly slower rate than that containing endogenous FKHR1. However, the nuclear protein(s) that bound the −1532 sequence migrated at the same rate as those that bound a known FKHR1-binding sequence from the IGFBP-1 promoter (IRS) (Fig. 7C) (28).

Also consistent with the involvement of FKHR1, the intensity of band A decreased in LPS/IFN-γ-stimulated cells, presumably due to PKB-dependent phosphorylation and exit of FKHR1 from the nucleus (31). Furthermore, wortmannin increased binding in basal and LPS/IFN-γ-stimulated cells, indicating inhibitory control by PI3K. Binding of endogenous nuclear FKHR1 to the 32P-labeled probe containing the −1532 site was efficiently competed by the addition of corresponding cold probe (Fig. 7C). Surprisingly, the intensity of the GST-FKHR1 (200 ng of protein)-shifted band was less than...
that of endogenous protein, perhaps due to the absence of binding cofactors or post-translational modification, or possibly resulting from interference by GST. In summary, the consensus FKHR1-binding sequence at the −1532 position in the hiNOS promoter mediates PI3K-dependent suppression of hiNOS transcription and binds FKHR1 in nuclear lysates from intact cells.

**DISCUSSION**

Via activation of cell-surface receptors, bacterial products and IFN-γ initiate local inflammatory responses that are essential components of innate immunity (40). *Escherichia coli* LPS binds to the toll-like receptor 4 and initiates a signal transduction cascade that, in part, leads to the transcription of pro-inflammatory genes (41). Synthesis of iNOS mRNA is a critical initial step in the production of NO in response to LPS and IFN-γ (6). NO results in DNA damage, killing of microorganisms, and modulation of immune effector cell function (42). In addition, NO can regulate cellular apoptosis or proliferation, responses that play important roles in tissue injury and the resolution of inflammation (8, 43).

Human iNOS transcription is strongly induced via binding of mitogen- and cytokine-activated transcription factors (e.g. STAT1, AP-1, and NF-κB) to the iNOS gene 5′-flanking region (10, 13, 15, 44). In part due to more subtle changes in gene expression, the identification of signaling mechanisms that simultaneously dampen transcriptional responses has been more challenging. For instance, an RNA interference-based genome screen in *Drosophila melanogaster* was used to identify Dnr1, a putative ubiquitin ligase, that suppresses basal Relish (NF-κB) activity (45). The observation that PI3K activity attenuated the induction of iNOS in mammalian cells (25) suggested that downstream protein kinases might play a simultaneous inhibitory role in the transcriptional response to mediators of innate immunity. In the current study, the transcription factor FKHR1 regulated hiNOS promoter activity in PI3K- and PKB-dependent fashion via binding to a specific sequence in the hiNOS promoter. Consistent with PI3K-dependent inhibition of iNOS induction (16, 24, 35, 36), LPS led to the phosphorylation of FKHR1, a modification known to inhibit FKHR1 transcriptional activity, and block the induction of FKHR1-dependent genes. By inhibiting PI3K or PKB, a 2- to 3-fold increase in hiNOS promoter activity or mRNA levels could be achieved in lung epithelial cells exposed to LPS and IFN-γ. FKHR1, its transactivation domain, and its PKB phosphorylation sites were required for this effect.

In contrast to NF-κB, AP-1, or STAT1, FKHR1 was a positive regulator of basal hiNOS promoter activity in addition to potentiating induction by LPS/IFN-γ (Fig. 4, B and C). Consistent with this observation, FKHR1 was constitutively localized to the nucleus as assessed by immunofluorescence or gel shift analysis (31). PKB-dependent phosphorylation of FKHR1 initiated its nuclear export resulting in diminished induction of FKHR1-dependent promoters (31).

Binding of native FKHR1 to the hiNOS promoter was not necessary for hiNOS induction (Figs. 5 and 6). Despite phosphorylation of FKHR1, and reduced DNA binding in response to LPS (Figs. 2 and 7), sub-maximal activation of transcription could still be achieved. These data can be summarized by the model illustrated in Fig. 8. In the absence of LPS and IFN-γ, PI3K activity inhibits basal hiNOS transcription (Fig. 8A). Although wortmannin abolished PI3K-dependent phosphorylation, and increases binding to the −1532 site (Fig. 2, 7), small changes in hiNOS promoter activity reflect the absence of other stimulatory transcription factors, such as STAT1, NF-κB, or AP-1 (Fig. 8B). Administration of LPS/IFN-γ causes combinatorial activation of the hiNOS promoter by multiple transcription factors; however, LPS simultaneously leads to PI3K-dependent phosphorylation of FKHR1, thereby limiting maximal transcription (Fig. 8C). Full induction can occur upon inhibition of PI3K (Fig. 8D) and recruitment of FKHR1 to the hiNOS promoter. Thus, FKHR1 provides a molecular mechanism for limiting the induction of pro-apoptotic genes under basal conditions or in the context of inflammation.

The importance of distinct inhibitory transcriptional control mechanisms on the synthesis of hiNOS mRNA has been explored in recent studies. NF-κB-repressing factor suppressed hiNOS promoter activity via a binding site 6.7-kb upstream of
the transcription start site (46). Peroxisome proliferator-activated receptor-γ acted as a transcriptional co-repressor by inhibiting iNOS promoter induction in murine macrophages in a STAT1-dependent fashion (47). The current study defines a new mechanism by which hiNOS mRNA levels can be limited in response to inflammatory stimuli. Interestingly, however, we observed a discrepancy between regulation of hiNOS promoter activity and mRNA levels. That is, LPS/IFN-γ further potentiated hiNOS mRNA levels, but not promoter induction, in cells overexpressing constitutively active FKHRL1 (Fig. 6, B and C). One explanation is that other FKHRL1-sensitive transcriptional response elements operate in the endogenous 5'-flanking region (at least 16 kb (12)) that are absent in the transfected 8.3-kb PGL3 hiNOS construct. Alternatively, overexpression of constitutively active FKHRL1 might regulate hiNOS mRNA levels independent of transcriptional control, for instance, by altering levels of proteins involved in mRNA stability.

Apart from direct effects on FKHRL1, other PI3K-mediated signaling mechanisms can determine the mRNA levels of inflammatory genes. PI3K-dependent inhibition of p38 MAPK led to decreased cyclooxygenase-2 mRNA levels due to a decrease in mRNA stability (48). In human monocyte/macrophages, inhibition of PI3K led to increased NF-κB and AP-1 activity, via cross-talk with mitogen-activated protein kinase pathways, and by the inhibition of glycogen synthase kinase-β (49). PI3K decreased NF-κB translocation to the nucleus, perhaps leading to the suppression of iNOS promoter activity (16, 24). However, our experiments in LPS/IFN-γ-stimulated A549 cells failed to demonstrate an enhancing effect of PI3K inhibition on NF-κB binding or activity (Fig. 2, A and B).

Its regulation by FKHRL1 and the PI3K pathway links hiNOS to other pro-apoptotic or growth-arrest genes that can be transcriptionally regulated during inflammation. In our study, PKB phosphorylation sites in FKHRL1 were necessary for PI3K-dependent suppression of hiNOS promoter activity (Fig. 4B). In addition, a functional FKHRL1-binding site in the hiNOS promoter was identified (Figs. 6 and 7). In similar studies examining PI3K-dependent transcriptional control of apoptosis and cell proliferation, overexpression of FKHRL1 increased p27kip1, TRAIL, and FasL promoter activity (29, 30, 38). FKHRL1 interacted with SMAD4 to control transforming growth factor-β1-dependent activation of the p21^{cip1} promoter (50). Thus, FKHRL1 represents a molecular node for the integration of PI3K and other transcriptional control pathways that regulate cell fate.

The PI3K-dependent phosphorylation of FKHRL1 by LPS described here (Fig. 2) represents one potential mechanism that...
might limit DNA damage and apoptosis during the innate immune response. In another study, the p53 tumor suppressor protein inhibited iNOS induction by cytokines when cells were exposed to NO donors (51). Increased p53 activity was related to ataxia telangiectasia mutated (ATM)- and ataxia telangiectasia and RAD3-related (ATR)-mediated phosphorylation of p53 (52). Similarly, FKHRL1 induced G$_{2}$/M arrest and p53 (52).

Similarly, FKHRL1 was implicated in the cellular response to DNA damage. FKHRL1 induced G$_{2}$/M arrest and DNA repair by activating the transcription of the growth-arrest and DNA damage-response gene (GADD45) (53). Furthermore, consistent with regulation of genes involved in oxidant stress, FKHRL1 mediated hydrogen peroxide-dependent control of cat-
alase transcription and cell senescence (54). Transcription of the anti-oxidant selenoprotein P gene was also regulated by FKHRL1 (53). These studies establish FKHRL1 as a molecular bridge between oxidant stress and the control of cell proliferation or death.

Mutation of the hiNOS FKHRL1 binding site (−1532), overexpression of dominant negative FKHRL1, or overexpression of anti-FKHRL1 siRNA did not completely abolish potentiation of hiNOS promoter activity by wortmannin (Fig. 6D). Although this may be a function of residual binding of endogenous FKHRL1, we cannot exclude PI3K-independent mechanisms of FKHRL1 regulation. For instance, IκB kinase-β phosphorylated FKHRL1 at Ser-644, inhibited FKHRL1 activity, and promoted tumorigenesis (38), suggesting coordinate regulation at different sites in FKHRL1. In another study, serum- and glucocorticoid-inducible kinase preferentially phosphorylated the Ser-315 moiety, whereas PKB-dependent regulation of FKHRL1 in the control of hiNOS transcription by LPS-dependent activation of PI3K (39).

REFERENCES

1. Steuh, D. J., and Griffith, O. W. (1992) Adv. Enzymol. Relat. Areas. Mol. Biol. 65, 287–346
2. Michel, T., and Feron, O. (1997) J. Clin. Invest. 100, 2146–2152
3. Lowenstein, C. J., Dinerman, J. L., and Snyder, S. H. (1994) Ann. Intern. Med. 120, 227–237
4. Nathan, C., and Xie, Q. W. (1994) Cell 78, 915–918
5. Schmidt, H. H., and Walter, U. (1994) Cell 78, 919–925
6. Nathan, C. (1997) J. Clin. Invest. 100, 2417–2423
7. Murphy, M. P. (1999) Biochim. Biophys. Acta 1411, 401–414
8. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Cell Death Differ. 6, 33361–33368
9. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Cell. Signal. 3, 233–239
10. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) Annu. Rev. Bio-
11. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1054–1059
12. Marks-Konczalik, J., Chu, S. C., and Moss, J. (1998) J. Biol. Chem. 273, 22201–22208
13. Marks-Konczalik, J., Chu, S. C., and Moss, J. (1998) J. Biol. Chem. 278, 33637–33644
14. Marks-Konczalik, J., Chu, S. C., and Moss, J. (1998) J. Biol. Chem. 273, 6482–6487
15. Marks-Konczalik, J., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 33361–33368
16. Marks-Konczalik, J., Chu, S. C., and Moss, J. (2003) J. Biol. Chem. 278, 137–148
17. Marks-Konczalik, J., Chu, S. C., and Moss, J. (2004) J. Biol. Chem. 279, 1716–1722
18. Kristof, A. S., Marks-Konczalik, J., Billings, E., and Moss, J. (2003) J. Biol. Chem. 278, 22201–22208
19. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
20. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
21. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
22. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
23. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
24. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
25. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
26. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
27. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
28. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
29. Kristof, A. S., Marks-Konczalik, J., and Moss, J. (2001) J. Biol. Chem. 276, 8445–8452
30. Ghaffari, S., Jagani, Z., Kitidis, C., Lodish, H. F., and Khosravi-Far, R. (1998) J. Biol. Chem. 273, 6482–6487
31. Dijkstra, P. F., Medema, R. H., Lamers, J. W., Koenderman, L., and Burgering, B. M., Raaijmakers, J. A., Lammers, J. W., Koenderman, L., and Burgering, B. M. (1998) Cell 96, 975–986
32. Dijkstra, P. F., Medema, R. H., Lamers, J. W., Koenderman, L., and Burgering, B. M. (1998) Cell 96, 975–986
33. Dijkstra, P. F., Medema, R. H., Lamers, J. W., Koenderman, L., and Burgering, B. M. (1998) Cell 96, 975–986
34. Dijkstra, P. F., Medema, R. H., Lamers, J. W., Koenderman, L., and Burgering, B. M. (1998) Cell 96, 975–986
35. Dijkstra, P. F., Medema, R. H., Lamers, J. W., Koenderman, L., and Burgering, B. M. (1998) Cell 96, 975–986
36. Dijkstra, P. F., Medema, R. H., Lamers, J. W., Koenderman, L., and Burgering, B. M. (1998) Cell 96, 975–986
37. Dijkstra, P. F., Medema, R. H., Lamers, J. W., Koenderman, L., and Burgering, B. M. (1998) Cell 96, 975–986
FKHRL1 Regulates the Human iNOS Promoter

2905–2927
38. Hu, M. C., Lee, D. F., Xia, W., Golfman, L. S., Ou-Yang, F., Yang, J. Y., Zou, Y., Bao, S., Hanada, N., Sasao, H., Kobayashi, R., and Hung, M. C. (2004) Cell 117, 225–237
39. Brunet, A., Park, J., Tran, H., Hu, L. S., Hemmings, B. A., and Greenberg, M. E. (2001) Mol. Cell. Biol. 21, 952–965
40. Trinchieri, G. (2003) Nat. Rev. Immunol. 3, 133–146
41. Beutler, B., Hoebe, K., Du, X., and Ulevitch, R. J. (2003) J. Leukocyte Biol. 74, 479–485
42. Nathan, C., and Shiloh, M. U. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8841–8848
43. Taylor, B. S., de Vera, M. E., Ganster, R. W., Wang, Q., Shapiro, R. A., Morris, S. M., Jr., Billiar, T. R., and Geller, D. A. (1998) J. Biol. Chem. 273, 15148–15156
44. Foley, E., and O’Farrell, P. H. (2004) PLoS Biol. 2, E203
45. Feng, X., Guo, Z., Nourbaksh, M., Hauser, H., Ganster, R., Shao, L., and Geller, D. A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14212–14217
46. Chen, C. W., Chang, Y. H., Tsi, C. J., and Lin, W. W. (2003) J. Immunol. 171, 979–988
47. Chen, C. W., Chang, Y. H., Tsi, C. J., and Lin, W. W. (2003) J. Immunol. 171, 979–988
48. Monick, M. M., Robeff, P. K., Butler, N. S., Flaherty, D. M., Carter, A. B., Peterson, M. W., and Hunninghake, G. W. (2002) J. Biol. Chem. 277, 32992–33000
49. Guha, M., and Mackman, N. (2002) J. Biol. Chem. 277, 32124–32132
50. Seoane, J., Le, H. V., Shen, L., Anderson, S. A., and Massague, J. (2004) Cell 117, 211–223
51. Forrester, K., Amba, S., Lupold, S. E., Kapust, R. B., Spillare, E. A., Weinberg, W. C., Felley-Bosco, E., Wang, X. W., Geller, D. A., Tzeng, E., Billiar, T. R., and Harris, C. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2442–2447
52. Hofseth, L. J., Saito, S., Hussain, S. P., Espey, M. G., Miranda, K. M., Araki, Y., Jhappan, C., Higashimoto, Y., He, P., Linke, S. P., Quezado, M. M., Zurer, I., Roter, V., Wink, D. A., Appella, E., and Harris, C. C. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 143–148
53. Tran, H., Brunet, A., Grenier, J. M., Datta, A. J., Jr., DiStefano, P. S., Chiang, L. W., and Greenberg, M. E. (2002) Science 296, 530–534
54. Nemoto, S., and Finkel, T. (2002) Science 295, 2450–2452
55. Motta, M. C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bulsma, Y., McBurney, M., and Guarente, L. (2004) Cell 116, 551–563