Cell Death and Differentiation (2003) 10, 508–515

www.nature.com/cdd

Inhibition of hypoxia/reoxygenation-induced oxidative stress in HGF-stimulated antiapoptotic signaling: role of PI3-K and Akt kinase upon rac1

M Ozaki*[,1], S Haga[1], HQ Zhang[1], K Irani[2] and S Suzuki[1]

[1] Bioengineering Laboratory, Department of Innovative Surgery, National Research Institute for Child Health and Development, Tokyo, Japan
[2] Division of Cardiology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA
* Corresponding author: M Ozaki, Bioengineering Laboratory, Department of Innovative Surgery, National Research Institute for Child Health and Development, 3-35-31, Taishi-Do, Setagaya, Tokyo 154-8567, Japan. Tel: +81 3 3416 0181_Ext. 8774; Fax: +81 3 3411 7309; E-mail: mozaki@nch.go.jp

Received 18.3.02; accepted 19.8.02
Edited by Nunez

Abstract

Rac1-regulated reactive oxygen species (ROS) production has been implicated in apoptosis. In contrast, pleiotropic protein kinase Akt protects against apoptosis. However, the pro- and antiapoptotic mechanisms of rac1 and Akt, respectively, and the intersection between these mechanisms are incompletely understood. In a model of oxidative stress and apoptosis induced by hypoxia/reoxygenation (H/R) in primary hepatocytes, activation of the PI3–K Akt axis by the prosurvival hepatocyte growth factor (HGF) inhibited H/R-stimulated rac1 activation and intracellular ROS production, and suppressed apoptosis. Suppression of PI3-K or Akt activity abrogated the inhibitory effect of HGF on rac1 activity and rac1-regulated oxidative stress. Furthermore, constitutive activation of Akt or PI3-K in the absence of HGF was sufficient to phosphorylate rac1, inhibit rac1 activation, and suppress rac1-regulated ROS production. These findings demonstrate that growth factor-stimulated activation of PI3-K–Akt is necessary and sufficient to suppress intracellular oxidative stress and apoptosis by inhibiting activation of pro-apoptotic, prooxidative rac1 GTPase.
Cell Death and Differentiation (2003) **10,** 508–515. doi:10.1038/sj.cdd.4401172

Keywords: rac1; ROS; PI3-K; Akt; HGF; hypoxia; reoxygenation; apoptosis

Abbreviations: ROS, reactive oxygen species; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; HGF, hepatocyte growth factor; 8-OHdG, 8-hydoxy-2-deoxyguanosine; NAC, *N*-acetyl cysteine; CM-H_2DCF-DA, 5,6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate

Introduction

Reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide are generated in many physiological and pathophysiological conditions.[1–5] There are accumulating data indicating that regulation of the intracellular redox state is a versatile control mechanism in signal transduction in both pathological and physiological conditions.[6]

Ischemia/reperfusion (I/R) and hypoxia/reoxygenation (H/R) result in the production of ROS within tissue or cells.[2,7,8] The rac1 small GTPase is an important regulator of ROS production within cells under these circumstances.[7] Rac1 belongs to the rho family of small GTP binding proteins and its role in the production of ROS in phagocytic cells such as neutrophils is well established.[9–11] In such cells, rac proteins are essential for the assembly of the plasma membrane NADPH oxidase, which is responsible for the transfer of electrons to molecular oxygen leading to the production of superoxide anions. Rac proteins, in particular rac1, function similarly in nonphagocytic cells,[1,12] and such rac1-regulated ROS have been implicated in a variety of cellular processes including growth, migration, and transformation.[2,8,12–17] We have recently shown that rac1-regulated ROS production also mediates apoptosis in response to I/R.[7]

Hepatocyte growth factor (HGF), which was initially isolated as a potent mitogen for hepatocytes, is now known to be a broad-spectrum mitogen for a variety of cell types.[18–22] In addition to these activities, HGF has morphogenic and angiogenic activities,[23–25] and is involved in organ regeneration and tumorigenesis.[26,27] HGF, after binding to its specific receptor tyrosine kinase (cMet), induces various biological effects mainly through activation of PI3-K and Akt kinases.[28–32] In this respect, HGF is a prototypical prosurvival growth factor. In support of this, HGF is also known to prevent nontransformed hepatocytes from oxidant-mediated apoptosis.[33,34]

Although the prosurvival effects of the PI3-K–Akt pathway are well known, the roles of PI3-K or Akt in regulating intracellular oxidative stress, if any, are only beginning to be appreciated. In this report, we describe a hitherto unknown role of Akt in suppressing ROS production, and show that Akt-induced phosphorylation with subsequent inhibition of rac1 activity is responsible for this phenomenon.

Results

HGF protects hepatocytes from H/R-induced apoptosis through PI3-K and Akt

We first validated the antiapoptotic role of HGF in our model of H/R-induced apoptotic cell death. Cellular apoptosis increased following H/R (Figure 1a). HGF effectively suppressed H/R-induced apoptosis. The maximum

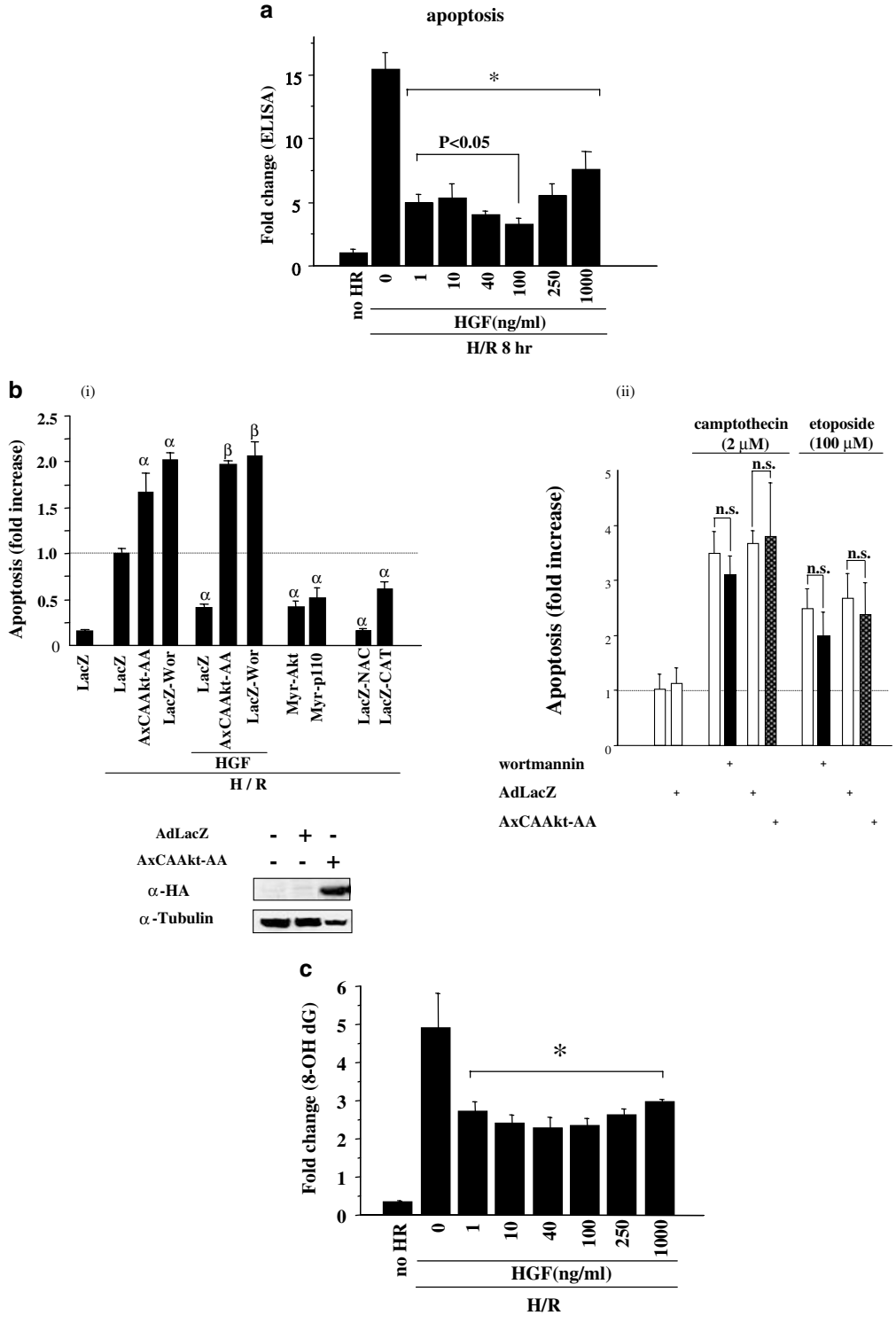

Figure 1 HGF prevents hepatocytes from apoptotic cell death through PI3-K and Akt. (**a**) Effects of HGF upon hepatocellular apoptosis induced by 4 h hypoxia and 8 h reoxygenation. *$P<0.05$ *versus* HGF(−)-H/R(+) group. (**b**) Effects of PI3-K and Akt upon suppressive effects of HGF against H/R-induced apoptosis. Culture media collected to evaluate apoptosis 8 h after hypoxia were applied for the assays. rhHGF, wortmannin (Wor), *N*-acetyl cysteine (NAC), or catalase (CAT) were administered 2 h prior to hypoxia at concentrations of 40 ng/ml, 0.2 μM, 1 mM, and 1000 U/ml, respectively. α: $P<0.05$ *versus* AdLacZ-HGF(−)-H/R(+) group, β: $P<0.05$ *versus* AdLacZ-HGF(+)-H/R(+) group, Expression of dominantly negative mutant of Akt protein was confirmed by Western blotting. To show the specific inhibitory effects of wortmannin and dominant negative mutant of Akt upon PI3-K and Akt, respectively, apoptosis was measured 8 h after the administration of the PI3-K/Akt-independent apoptosis inducers, camptothecin or etoposide (2 and 100 μM, respectively). (**c**) Effects of HGF upon the generation of 8-OHdG (oxidative addicts of dsDNA) induced by 4 h hypoxia and 8 h reoxygenation. *$P<0.05$ *versus* HGF(−)-H/R(+) group. Each experiment was performed at least three times independently. All data are expressed as mean ± S.E.M

effects of HGF were obtained around 40–100 ng/ml in all groups.

We next determined whether the antiapoptotic effect of HGF was mediated by Akt kinase and PI3-K. HGF-stimulated protection from apoptosis was completely inhibited by adenoviral expression of a dominant-negative mutant of Akt kinase (AxCAAkt-AA) as well as a specific PI3-K inhibitor, wortmannin. Furthermore, overexpressed constitutively activated mutants of Akt or PI3-K (Myr-Akt or Myr-p110, respectively) protected H/R-induced apoptosis (Figure 1b-i). The specific inhibition of PI3-K/Akt pathway by AxCAAkt-AA or wortmannin was confirmed by PI3-K/Akt-independent apoptosis inducers, camptothecin and etoposide. Camptothecin and etoposide, proapoptotic factors independent of PI3-K/Akt pathway, which inhibit topoisomerase I and II respectively, induced apoptosis of primary hepatocytes. Pretreatment of wortmannin and AxCAAkt-AA, however, did not have any effects upon camptothecin- and etoposide-induced apoptosis (Figure 1b-ii).

These findings show that Akt and PI3-K function as mediators of HGF-stimulated protection against H/R-induced apoptotic cell death via suppression of intracellular oxidative stress, because the antioxidants *N*-acetyl cysteine (NAC) and catalase also significantly reduced H/R-induced apoptosis.

HGF inhibits H/R-induced oxidative stress and apoptosis

Intracellular redox changes closely accompany H/R-induced cell death. We therefore also examined the role of HGF in modulating H/R-stimulated oxidative stress in hepatocytes. HGF reduced H/R-induced intracellular ROS accumulation (Figure 4d, f), as well as generation of 8-hydoxy-2-deoxyguanosine (8-OHdG), a marker of oxidative stress (Figure 1c). This suggests that HGF exerts its protective effect upon H/R-induced apoptosis via suppression of intracellular oxidative stress.

Preactivation of Akt is required for HGF-induced cytoprotection

With an eye toward examining the mediating role of Akt in HGF-stimulated suppression of oxidative stress, we next determined whether HGF results in Akt activation. Akt was activated by HGF 30 min after administration, and this effect was inhibited by pretreatment with PI3-K inhibitors, Ly294002 or wortmannin. Sufficient inhibitory effect of Akt kinase activity was obtained by Ly294002 at 10 and 50 μM, and by wortmannin at 200 nM (Figure 2a). This suggests that stimulation with HGF results in activation of Akt through PI3-K, and that this activation may be required to protect against apoptosis. In support of this, HGF, when administered immediately following hypoxia, did not significantly suppress apoptosis induced by H/R (Figure 2b).

HGF suppresses rac1-regulated, H/R-induced ROS production through activation of Akt (Figures 3 and 4)

We then examined the role of HGF in modulating ROS production, particularly that regulated by rac1. Adenoviral

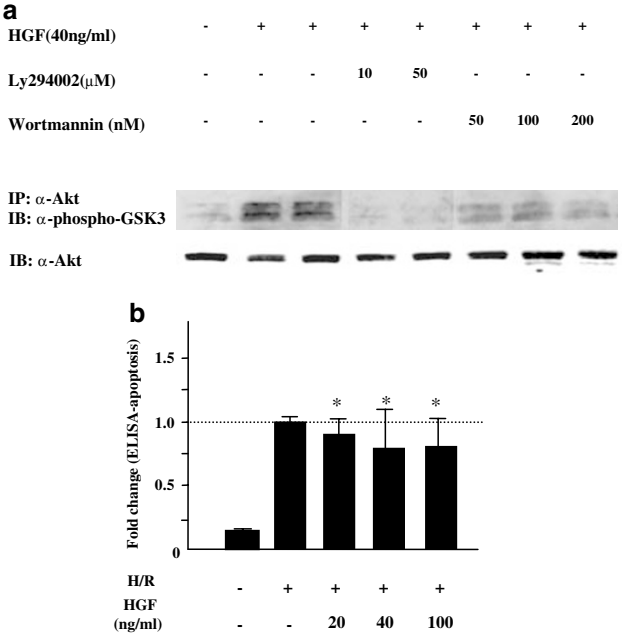

Figure 2 Preactivation of Akt is required for HGF-induced cytoprotection. (**a**) Activation of Akt by HGF. Akt activity was measured with or without addition of HGF. Whole-cell extracts were prepared from 3×10^6 cells, and immunoprecipitated with anti-Akt. After GSK-3, a substrate of Akt, was added to the reaction mixture, phosphorylation of GSK-3 was measured with anitphospho-GSK-3. (**b**) HGF's effects upon posthypoxic apoptotic cell death, when administered following hypoxia. rhHGF was administered just following hypoxia at the concentrations indicated, and apoptotic cell death was measured 8 h following hypoxia. *n.s. *versus* HGF(−)-H/R(+) group

expression of an activated allele of rac1 (rac1V12) resulted in an increase in intracellular ROS levels. Treatment with HGF suppressed this rise in rac1-regulated ROS in hepatocytes (Figure 3b, c). Treatment with HGF alone did not induce ROS production, nor did it result in an increased capacity to eliminate ROS directly, as determined by measuring intracellular ROS after addition of exogenous peroxide (Figure 4b, c).

We next examined whether Akt mediates HGF-stimulated suppression of intracellular ROS production. As shown in Figure 4, pretreatment with HGF inhibited H/R-induced ROS production. More importantly, this effect of HGF was abolished by adenoviral expression of dominant-negative mutants of PI3-K (AxCAdelta-p85) or of Akt (AxCAAkt-AA). As expected, H/R-induced oxidative stress was also suppressed by dominant-negative rac1 (racN17) and NAC. This suggests that Akt and PI3-K are essential mediators through which HGF inhibits oxidative stress.

We also examined whether activated Akt or PI3-K could directly affect the intracellular redox state and, specifically, rac1-regulated ROS production, independent of HGF. As demonstrated previously, expression of the activated allele of rac1 (rac1V12) resulted in an increase in oxidative stress. However, coexpression of rac1V12 with constitutively activated forms of PI3-K (Myr-p110) or Akt (Myr-Akt) inhibited this rise in intracellular ROS (Figure 3d, e). This shows that active Akt or PI3-K is sufficient to suppress intracellular oxidative stress, and acts by inhibiting rac1-regulated ROS production.

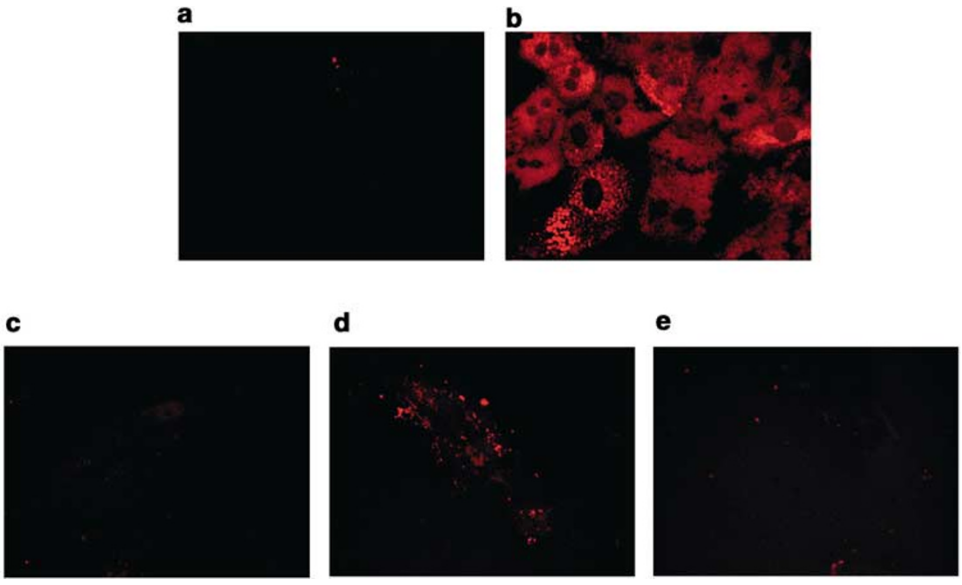

Figure 3 rac1-mediated ROS production was diminished by HGF, and by overexpressed constitutively activated PI3-K and Akt. ROS production of the hepatocytes was observed with confocal laser scan microscopy after staining cells with CM-H_2DCF-DA (250 μM) for 10 min. Adenovirus encoding constitutively activated mutant of rac1 (Adrac1V12) was infected 48 h prior to the experiment (**b**–**f**). (**a**) Hepatocytes infected with AdLacZ. (**b**) ROS production by constitutively activated rac1 (racV12). (**c**) 20 min after HGF addition. (**d**) Coinfected with constitutively activated mutant of PI3-K (Myr-p110). (**e**) Coinfected with constitutively activated mutant of Akt (Myr-Akt). Each photograph taken in this figure includes at least several cells and expresses a representative of at least three independent experiments. Original magnification, $\times$ 200

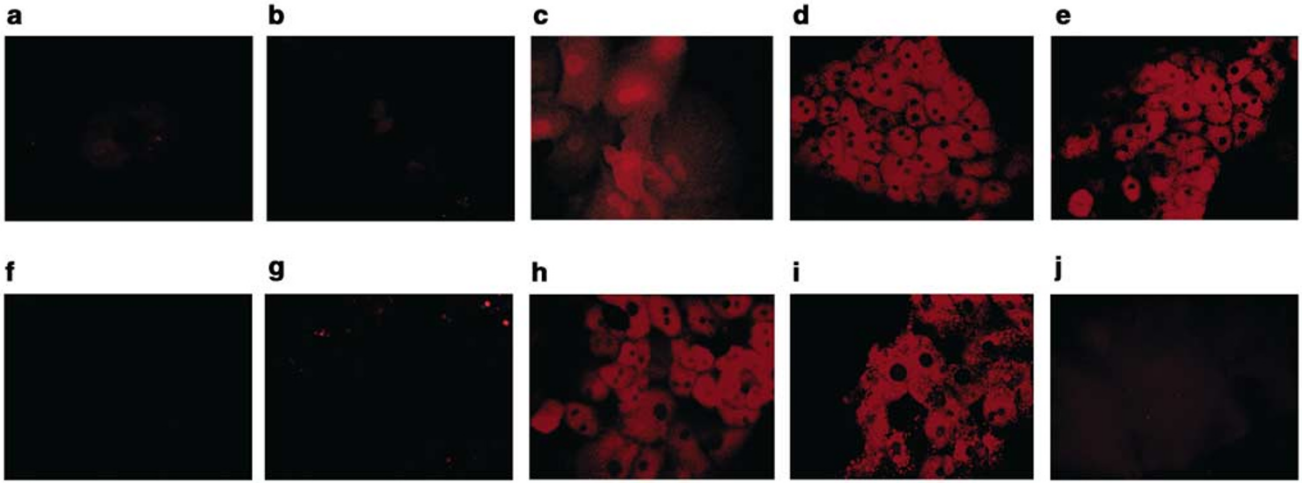

Figure 4 Measurement of cellular ROS using confocal laser scan microscopy. Hepatocytes are reoxygenated and stained with 10 μM of CM-H_2DCFDA 10 min before observation. Adenoviruses (AxCAdelta-p85 or AxCAAkt-AA, dominantly negative mutant of PI3-K or Akt, respectively) were infected 48 h prior to experiment. HGF was added to the culture medium 2 h prior to hypoxia. (**a**) Normoxia. (**b**) 10 min after HGF addition (40 ng/ml). (**c**) 10 min after addition of HGF and H_2O_2 (100 μM). (**d**) H/R only (10 min reoxygenation following 4 h hypoxia). (**e**) H/R preinfected with AdLacZ. (**f**) H/R pretreated with HGF. (**g**) H/R preinfected with AdracN17 (dominant negative mutant of rac1). (**h**, **i**) H/R pretreated with HGF, and preinfected with AxCAdelta-p85 or AxCAAkt-AA, respectively. (**j**) H/R pretreated with NAC (10 mM). Each photograph expresses a representative of at least three independent experiments. Original magnification, $\times$ 200

HGF phosphorylates and inactivates rac1

To further elucidate the molecular mechanisms responsible for the above-mentioned phenomena, we examined the effect of HGF and Akt on (1) rac1 activity, and (2) post-translational modification of rac1. Rac1 was activated within minutes after H/R (Figure 5a). Pretreatment with HGF reduced H/R-induced rac1 activation (Figure 5b). This inhibitory effect of HGF on posthypoxic rac1 activity was abolished by suppressing PI3-K or Akt activation. Consistent with these findings, expression of constitutively active PI3-K or Akt (Myr-p110 or Myr-Akt, respectively) also suppressed posthypoxic rac1 activation (Figure 5c). These observations indicate that HGF inhibits activation of rac1 through the PI3-K-Akt pathway.

A recent report shows that rac1 has a putative Akt phosphorylation site, and that serine phosphorylation of rac1 by Akt results in inhibition of GDP–GTP exchange *in vitro*.[35] If true *in vivo*, this could provide the molecular basis for the inhibitory effect of HGF, via Akt and PI3-K, on rac1 activity. We therefore examined whether HGF modulates serine phos-

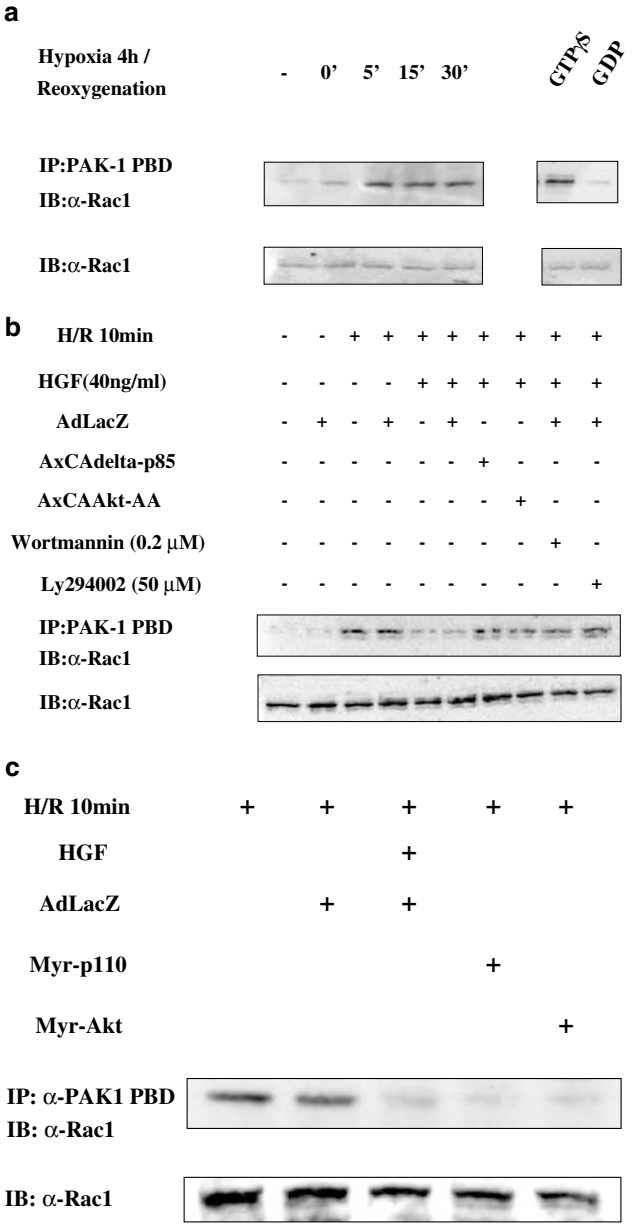

Figure 5 HGF inactivates rac1 through PI3-K and Akt. (**a**–**c**) rac1 activity assay. (**a**), rac1 is activated following hypoxia and H/R. Whole-cell extracts were immunoprecipitated with p21-binding domain of PAK-1 (PAK-1 PBD) and blotted with anti-rac1. (**b**) Suppression of H/R-induced rac1 activity by HGF through PI3-K and Akt. (**c**) Suppression of H/R-induced rac1 activation was abolished completely by HGF, Myr-p110, and Myr-Akt

phorylation of rac1. Rac1 was constitutively serine phosphorylated under basal conditions, and H/R resulted in dephosphorylation of rac1. Treatment with HGF phosphorylated rac1 even under normoxic conditions, and inhibited H/R-stimulated serine dephosphorylation of rac1 (Figure 6a). This suggests that HGF inhibits activation of rac1 by maintaining it in the phosphorylated form (and thus suppressing GDP–GTP exchange), an effect that may be mediated via Akt. Furthermore, overexpression of constitutively activated mutants of PI3-K or Akt also inhibited H/R-stimulated serine dephosphorylation of rac1 (Figure 6b). These data suggest

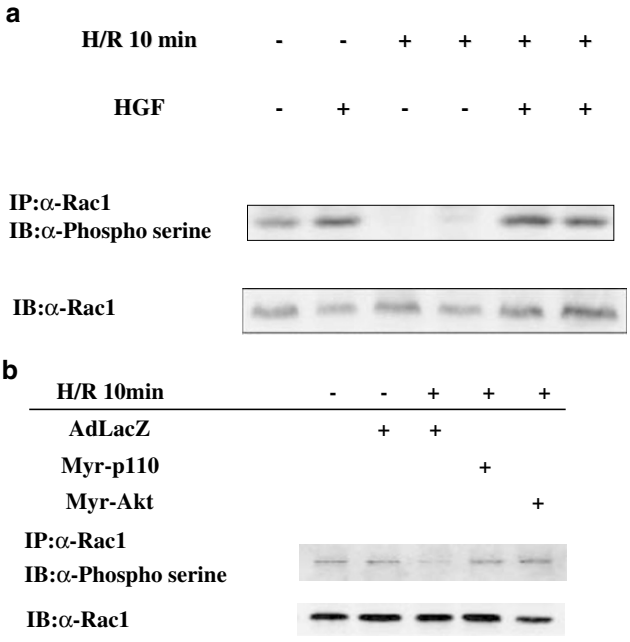

Figure 6 rac1 phosphorylation assay. Treatment with HGF (**a**) and constitutively activated mutants of PI3-K or Akt (**b**) phosphorylate rac1 in normoxic conditions and prevent serine dephosphorylation of rac1 after H/R. Whole-cell extracts were immunoprecipitated with anti-rac1 and blotted with antiphosphoserine. Each assay expresses the representative of three independent experiments

that HGF acts via the Akt–PI3-K axis to induce phosphorylation and suppress activation of rac1.

Discussion

Although the antiapoptotic effects of HGF are well known, our findings are the first to show that HGF suppresses apoptosis by inhibiting rac1-regulated oxidative stress. Moreover, our data show that activation of the PI3-K–Akt prosurvival pathway is essential and sufficient for HGF's effects on rac1, and rac1-regulated ROS production.

Interestingly, HGF itself does not induce any changes in the intracellular redox state. This is somewhat surprising since many other receptor tyrosine kinase-linked growth factors such as PDGF and EGF, and cytokines such as TNF-α and IL-1β employ ROS as signaling intermediaries.[36–38] For a growth factor, HGF seems to function in a unique fashion in that it does not employ ROS as its own downstream signaling molecules, but rather inhibits one of the cellular machineries responsible for ROS generation. In this regard, the serine phosphorylation of rac1 by HGF provides a novel molecular mechanism for suppression of intracellular oxidative stress.

Rac1 is of growing importance in H/R and I/R settings. Somewhat paradoxically, activation of rac1 in such pro-oxidative settings is closely coupled to activation of Akt. How then could activation of the PI3-K–Akt axis result in down-regulating rac1 activity, and suppressing oxidative stress? The answer to this paradox lies in understanding that activation of PI3-K and Akt is a compensatory prosurvival

mechanism in response to oxidative stimuli. Preactivation of this mechanism, as with HGF or expression of activated alleles of Akt or PI3-K, before the oxidative stimulus, inhibits oxidative stress and offers protection from death induced by that stimulus. This compensatory mechanism, if activated after the oxidative stimulus and oxidative stress, may act to suppress subsequent ROS production (by inhibiting rac1 function), but at that particular time is not sufficient to prevent cell death. This is borne out by our observations that (1) Akt was activated even in the setting of cell death induced by H/R (data not shown) and (2) HGF offered no protection against cell death if administered after the hypoxic stimulus.

It is also likely that, in contrast to the proapoptotic H/R stimulus, under more controlled conditions of ROS production such growth factors (angiotensin II, PDGF, EGF, etc.), rac1 and Akt have a more symbiotic relationship.[1,36,37,39,40]. In such scenarios, rac1 and rac1-regulated ROS, through activation of Akt, may promote growth and prevent cell death. In fact, such prosurvival effects of rac1-regulated ROS have been demonstrated.[12,38,41]

In summary, our findings implicate a novel relationship between the PI3-K–Akt axis and rac1 in the control of intracellular redox state. The balance of this relationship, controlled by prosurvival and prodeath stimuli such as HGF and H/R, respectively, and the timing between such stimuli, has a profound impact on the fate of the cell. Rac1, PI-3 K, and Akt are expressed and operative in all cell types, implying that the relationship described herein may be relevant in a broad variety of cell types and tissues.

Materials and Methods

Preparation of rat hepatocytes and H/R experiment

Rat hepatocytes were prepared freshly from Lewis rat (male, 250–300 g) by conventional collagenase method. The isolated hepatocytes were seeded in plastic dishes coated with rat-tail collagen (3×10^6 cells per 100 mm dish) and cultured with William's E complete medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum, 10^{-9} mol/l insulin, and 10^{-9} mol/l dexamethasone in a humidified atmosphere of 5% CO_2/95% air at 37°C. Medium was replaced by William's E medium without serum, insulin, and dexamethasone 16 h before the experiment. Hypoxic condition was attained in a modulator incubator chamber (Billups-Rothenberg, CA, USA) by flushing the chamber with a 95% N_2/5% CO_2 gas mixture for 10 min and sealing the chamber. This method has been shown to achieve a pO_2 of 10 ± 5 Torr. Following 4 h of hypoxia, reoxygenation of hepatocytes was obtained by opening the chamber and by replacing the hypoxic medium with oxygenated medium.

Adenoviral vectors and recombinant human HGF (rhHGF)

HA-epitope-tagged dominant negative mutant of Akt in which Thr308 and Ser473 are replaced by alanine (AxCAAkt-AA), a dominant negative mutant of the p85 subunit of PI3-K (AxCAdelta-p85), constitutively activated mutant of Akt (myristoylated Akt: Myr-Akt), and constitutively activated mutant of PI3-K (myristoylated p110: Myr-p110) were kindly provided by Dr. W Ogawa and Dr. M Kasuga (Kobe, Japan). An adenoviral vector encoding *Escherichia coli* lacZ gene (AdLacZ) was used as a control vector. Adenoviral vector was infected to hepatocytes at 5 moi 48 h prior to the experiment, and rhHGF provided by Mitsubishi Chemical Corporation (Yokohama, Japan) was administered to hepatocytes 2 h prior to hypoxic insult at the concentration of 40 ng/ml, unless indicated. rhHGF obtained was blotted with anti-α-chain of HGF (2 μg/ml, Santa Cruz Biotechnology Inc., CA, USA) to confirm the activated form. Catalase, wortmannin, and Ly294002 were purchased from Roche (Basel, Switzerland), and *N*-acetyl cysteine (NAC) from Sigma (MO, USA).

Analyses for apoptosis, oxidative damage, and Akt activities of hepatocytes

For evaluation of apoptosis, an enzyme-linked immunosorbent assay (ELISA) kit (Cell Death Detection ELISA^{PLUS}; Roche, Basel, Switzerland) was used according to the manufacturer's instructions. The principle of this test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates by using biotinylated antihistone- and peroxidase-coupled anti-DNA antibodies. Hepatocellular necrosis induced by H/R was estimated by measuring LDH levels of the culture medium. DNA-damage biomarker Kit '8-OHdG Check plus', purchased from Japan Institute for the Control of Aging (Shizuoka, Japan), was used for the evaluation of oxidative stress in hepatocytes. Aliquots of the culture media were used for the oxidative stress assay. 8-OHdG Check is a competitive *in vitro* ELISA for quantitative measurement of the oxidative DNA adducts, 8-OHdG, in DNA with very high sensitivity.

Akt activity assay was performed using the Akt kinase Assay Kit (Cell Signaling, MA, USA) according to the manufacturer's instructions. Whole-cell extracts from 3×10^6 hepatocytes were immunoprecipitated with immobilized α-Akt, and *in vitro* kinase assay was performed using GSK-3 fusion protein as a substrate according to the manufacturer's instructions. Phosphorylation of GSK-3 was detected with antiphospho-GSK-3.

Intracellular ROS measurement

Cells (1×10^4) of hepatocytes were seeded in 35 mm-glass bottom dish 24 h before the experiment when adenovirus was not to be infected. The adenoviruses were infected for 5 h in serum-free medium 48 h before H/R experiment, when necessary. ROS production of the hepatocytes was observed with confocal laser scan microscopy (Olympus, Tokyo, Japan) after staining cells with 5,6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA, 250 μM in HBSS) for 10 min.

rac1 activity and rac1 phosphorylation assays

rac1 activity was measured using the 'rac1 activation kit' (Upstate Biotechnology, NY, USA) with slight modification. Briefly, whole-protein extracts were immunoprecipitated with protein binding domain of p21 activation kinase-1 (PAK1-PBD). PAK1-PBD only binds to activated forms of rac1 and cdc42. Immunoprecipitated proteins were separated in SDS-PAGE (10%) and blotted with anti-rac1 (1 μg/ml, Santa Cruz Biotechnology Inc., CA, USA). For rac1 phosphorylation assay, whole cell extracts were immunoprecipitated with anti-rac1 (Santa Cruz Biotechnology Inc., CA, USA), separated in SDS-PAGE (10%) and blotted with antiphospho serine (Chemicon, CA, USA).

Statistical analysis

All results were expressed as mean $\pm$ S.E.M. of at least three independent experiments. A one-way ANOVA was used for multiple comparisons. A value of $P < 0.05$ was considered significant.

Acknowledgements

We thank W Ogawa and M Kasuga for providing adenoviral vectors (AxCAAkt-AA, AxCAdelta-p85, Myr-Akt, and Myr-p110), and T Ishii for recombinant human HGF. We also thank Miss Y Nagata for excellent technical support in confocal laser scan microscopy.

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