Endogenous Calcitonin Gene-related Peptide Protects Human Alveolar Epithelial Cells through Protein Kinase Cε and Heat Shock Protein*

Received for publication, December 9, 2004, and in revised form, February 28, 2005. Published, JBC Papers in Press, March 22, 2005, DOI 10.1074/jbc.M413864200

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The intracellular mechanisms of ischemic preconditioning (PC) in preventing lung dysfunction following transplantation, shock, and trauma remain poorly understood. Previously, we have shown that alveolar epithelial cells secrete calcitonin gene-related peptide (CGRP) under inflammatory stress. Using a hypoxia/reoxygenation (H/R) and PC model, we found that CGRP was also secreted from human type II alveolar epithelial cells (A549) after PC. The locally released CGRP interacted with its receptor on the membrane of A549 cells and elicited downstream signals mediating the PC effect, because hCGRPs−/− mice did not produce any CGRP response. This result indicates that CGRP not only protects alveolar epithelial cells from H/R injury, but also acts as a paracrine factor in maintaining the local homeostasis and integrity of alveoli.

As a widely existing neuropeptide, calcitonin gene-related peptide (CGRP) has broad regulatory effects throughout the body, especially in the cardiovascular and respiratory system (6, 7). In addition to its known effects on vasodilation (8), immunomodulation (9), and repression of vascular smooth muscle cell proliferation (10, 11), CGRP has also been implicated to have a protective role in preconditioning of isolated rat hearts and intestines (12, 13). Regarding the lung, both CGRP and its receptor are expressed in type II alveolar epithelial cells. The basal secretion of CGRP from these cells helps to retain airway homeostasis, whereas enhanced release of CGRP under inflammatory stress plays a negative feedback role in inhibiting local immunoreaction (7, 14). These lines of evidence indicate the diverse benefits of CGRP under both physiological and pathological conditions in alveoli. Whether locally released CGRP plays a role in lung I/R injury or participates in the protective effect of PC is unknown.

The present study was designed to evaluate the role of CGRP in lung I/R and PC with a hypoxia/reoxygenation (H/R) model in the type II alveolar epithelial cell line (A549) that is sensitive to ischemia. The results suggest an autocrine mechanism of CGRP mediating the PC effect in these cells. The post-receptor signaling of CGRP relies on activation of protein kinase Cε (PKCε), which led to the induced expression of heat shock protein 70 (HSP70).

It is well known that the lung is highly susceptible to ischemia/reperfusion (I/R) injury. Studies have highlighted several aspects of the pathological changes by I/R as well as targets for treatment, including inflammatory infiltration, functional impairment, and loss of cell viability (1). Although suppression of immune responses and improvement of alveolar fluid clearance has received much attention, only a few studies directly evaluated the I/R injury of alveolar epithelial cells, especially type II epithelial cells that have multiple roles in maintaining the local homeostasis and integrity of alveoli (2). Ischemic preconditioning (PC) is a genetically conservative, intrinsic, and self-protective process that effectively defends I/R injury in various systems, including the heart, kidney, and lung (1, 3). For instance, PC prevents cardiac myocytes from I/R-induced cell death through several well-defined pathways (4). Numerous exogenous and endogenous agents have been verified to mimic the PC effect on myocytes in vitro (5). These well-developed models and procedures can be transplanted to the field of lung I/R and PC research to promote these studies at the single cell and subcellular level.
CGRP Induces HSP70 through PKCs

EXPERIMENTAL PROCEDURES

Materials—Recombinant human αCGRP, hCGRP2-37, and anti-CGRP antibody were purchased from Peninsula Laboratories, Inc. (Belmont, CA). Calphostin C, Rp-cAMPs and H89 were from Calbiochem. Glycinebenclamide and 5-hydroxyecanoid acid were from Sigma. Myristoylated PKCε inhibitory peptide (PKCV1-2) was from Biomol International L.P. (Plymouth Meeting, PA). Enhanced chemiluminescence detection reagents were from Pierce. Anti-HSP70 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PKCα antibody was from Pharmingen. Anti-actin antibody was from Abcam (Cambridge, MA). Cell culture media and supplements were from Hyclone (South Logan, UT). The BBL™ GMPak™ anaerobic system was from BD Biosciences. This system relies on serial chemical reactions, which consume oxygen and release CO2, to achieve an anaerobic environment with 4–10% of CO2.

Cell Culture—A549 cells were a kind gift from Dr. Remick (Department of Pathology, University of Michigan Medical School). Dr. Remick purchased the cells from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For all experiments, cells were used at 70–80% confluence. The cells were incubated in fetal bovine serum-free medium for 1–2 h before being exposed to different chemicals.

Setting Up H/R and PC Models—Experiments were carried out in six groups (as shown in Fig. 1A) with: 1) normoxic cultured cells and 2) H/R cells incubated with Hanks’ solution (free of gas and glucose) in the anaerobic system for 6 h, followed by normoxic culture in fetal bovine serum-free Dulbecco’s modified Eagle’s medium for 24 h; 3) cells in the PC group first cultured in Hanks’ solution (free of gas and glucose) for 10 min, fetal bovine serum-free Dulbecco’s modified Eagle’s medium for 10 min, and then H/R treatment; 4) inhibitors added 30 min prior to PC treatment to block the effects of PC; 5) CGRP added 1 h before H/R treatment; and 6) inhibitors added 30 min before CGRP to block the effects of CGRP.

Inhibition of CGRP Expression by Small Interfering RNA (siRNA)—Because our previous results have shown that A549 cells expressed only β-CGRP (but not α-CGRP) (14), construction of an RNA interfering plasmid for the β-CGRP gene involved the use of a pSilencer plasmid containing the H1-RNA promoter in concert with a pair of 64 oligonucleotides, each containing the unique sequence of 19 nucleotides (the underlined sequence of sense and antisense orientation) derived from the human CGRP gene: forward oligo, 5′-GATCCCGCGGACGCTTGTGGTGACCTTGGCTACAAGGAGAATCAGCAGGGTGGCTTTTGAGAAA-3′; reverse oligo, 5′-GGTTTTTCCAAAAACACTGCGACCTGTGGAC-TTCTCTTGAAGTACGCTACGATGGCGCGATGTGTGGG-3′. The sequence for siRNA was empirically selected and underwent a BLAST software search to ensure no significant homology with other genes. These oligonucleotides were annealed and ligated into the vector between the BglII/HindIII sites and transferred to A549 cells with use of Lipofectin (Invitrogen). For stable inhibition of CGRP, 2 μg of pSilencer-CGRP was cotransfected with 1 μg of pBabe-puro plasmid. The transfected cells were selected with 1 μg/ml puromycin for 7 days. Monoclines were chosen and expanded. Reverse transcription-PCR and radioimmunoasay showed the decreased CGRP mRNA level and protein secretion by 50–70% in transfected cells (see supplemental Fig. 1). Stable Transfer of CGRP Gene into A549 Cells—A pDNA3.1 plasmid containing human CGRP cDNA was constructed as previously described (9) and transferred to A549 cells with use of Lipofectin. After transfection, the cells were cultured in the presence of G418 (800 μg/ml) for 4 days, and a stable transfected cell line was selected by subculturing the cells from a monolone.

Cell Viability Assay—The cell injury index was determined by measuring lactate dehydrogenase (LDH) release and counting trypan blue-positive cells. After 24 h of reoxygenation, LDH contents were analyzed in culture medium by use of a biochemical analyzer (7170A, Hitachi) or a cytotoxicity detection kit (Roche Applied Science). The cells were trypanized, stained with 0.04% trypan blue, and counted using a cytometer to measure the living cells, dead cells, and the mortality rate.

Radioimmunoassay—Radioimmunoassay of CGRP was carried out following a procedure previously used in our laboratory (15). Briefly, the culture supernatants were collected and vacuum-dried. Samples were incubated with anti-CGRP antibody at 4 °C for 24 h. After the addition of 125I-labeled CGRP, samples were further incubated at 4 °C for 24 h. The bound radioactivity of calibrators, controls, and samples was measured in duplicate with use of a γ scintillation counter (Beckman Instruments).

Western Blotting—Cells were lysed, homogenized by ultrason and, boiled for 5 min. Following quantification of the protein concentration using the bicinchoninic acid method (Pierce), cell extracts were loaded onto 10% bis-acrylamide gels and separated by SDS-PAGE. Separated proteins were transferred from gels to polyvinylidene difluoride membranes and underwent immunoblotting to reveal HSP70 proteins using anti-HSP70 antibody, horseradish peroxidase-conjugated secondary antibody, and chemiluminescence. The protein bands were quantitatively analyzed using Kodak 1D image analysis software.

For membrane protein extraction, whole cell lysate was centrifuged at 8000 × g for 10 min. The supernatant was further centrifuged at 20,000 × g for 45 min. The supernatant after the second centrifugation was used as a cytosol fraction. The pellet was resuspended in lysis buffer containing 50 mM Tris-Cl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 0.1% Nonidet P-40 and was used as a membrane fraction. Western blotting was carried out with mouse anti-PKCε antibody (1:500–1000).

Statistics—The results are expressed as means ± S.E. Data analysis involved use of GraphPad Prism software. One-way analysis of variance, Student-Newman-Keuls test (comparisons between multiple groups), or unpaired Student’s t test (between 2 groups) was employed as appropriate. A p < 0.05 was considered significant.

RESULTS

Setting Up H/R and Preconditioning Models—A cellular model of H/R and PC was created by use of the anaerobic system and multiple procedures as described under “Materials and Methods” and Fig. 1A. A549 cells were exposed to prolonged hypoxia for 6 h followed by reoxygenation for 24 h (H/R).
Cell injury was revealed by increased LDH content in the culture medium, double the number of trypan blue-positive cells and profound cell morphological changes, including cell shrinkage, detachment, and fragmentation after H/R (Fig. 1, A and C). PC treatment (10-min hypoxic and 10-min normoxic culture prior to H/R) efficiently prevented A549 cells from H/R-induced cell damage (Fig. 1, A and C).

Role of Endogenous CGRP in PC—Previous studies have shown that CGRP content was increased in ischemic and reperfused tissues, which suggests that endogenous CGRP may play a role in ischemia and reperfusion (16). We carried out experiments to detect CGRP secretion in A549 cells during a broad time scale after H/R and PC. The basal level of CGRP in the culture supernatant of A549 cells was 40 pg/ml and remained stable during normoxic culture. The CGRP secretion was increased after hypoxia and remained at a high level during 24-h reoxygenation (data not shown). More importantly, PC also induced CGRP secretion in A549 cells, which persisted up to 24 h (Fig. 2A).

Because the CGRP receptor is also expressed on the membrane of A549 cells (14), we tested whether this secreted CGRP played a role in PC. Pre-incubation of hCGRP_{8–37} (100 nM), a CGRP receptor antagonist, significantly attenuated the protective effect of PC (Fig. 2B). These data indicate that CGRP is secreted in response to PC, and the endogenous CGRP mediates, at least in part, the protective effect of PC against H/R injury in A549 cells.

To confirm that the endogenous CGRP has cell protective effects, we used molecular and genetic manipulation of the CGRP expression. Knock down of CGRP mRNA by siRNA specifically attenuated both the CGRP mRNA level in A549 cells and protein secretion in the culture supernatant to 30–50% of that of controls, without influencing the expression of other related peptides, adrenomedullin, amylin, and calcitonin (see supplemental Fig. 1). Inhibition of CGRP expression by siRNA exacerbated H/R-induced cell damage in A549 cells (Fig. 3A). However, A549 cells stably transfected with the CGRP gene were resistant to H/R-induced cell death (Fig. 3B). Consistent with the results in Fig. 2B, these data support a cell-protective effect of endogenous CGRP against H/R injury.

Exogenous CGRP Mimicked the Effect of PC—to study the causal role of CGRP in cell protection further, we used a bioactive CGRP peptide to preincubate A549 cells before H/R. As revealed by LDH release and cell counting, preincubation with CGRP (10–100 nM) for 1 h reduced the cell damage induced by subsequent H/R. This effect was comparable with the protective effect of PC and was sensitive to hCGRP_{8–37} (1 μM) (Fig. 4). These data suggest that exogenous CGRP mimics the PC effect that protects A549 cells against H/R injury through the CGRP receptor.

CGRP Up-regulated HSP70 Expression in A549 Cells—Heat shock proteins (HSPs), a group of inducible chaperones, are involved in responses to various stresses, including H/R (17). The PC-induced expression of HSP70 is a major downstream event leading to cell protection against H/R injury (18). Therefore, we used HSP70 expression to evaluate the mechanism of the role of CGRP in H/R and PC further. As shown in Fig. 5A, both PC and bioactive CGRP (100 nM) doubled the protein level of HSP70 as compared with normoxia controls or cells under H/R. CGRP interfering RNA lowered the HSP70 protein level in normal cultured A549 cells (data not shown) and in cells after H/R treatment (Fig. 5B), whereas overexpression of the CGRP gene increased HSP70 expression in normoxic cultured cells (data not shown) and in cells under H/R (Fig. 5C). Thus, CGRP directly up-regulates HSP70 expression, which may explain the mechanism of CGRP in cell protection against H/R injury.

PKC and Mitochondrial K<sup>+</sup>ATP Channel-mediated CGRP Effects—To elucidate the intracellular signaling pathway responsible for the effects of CGRP on cell protection and HSP70 expression, various pathways that may participate in PC, such as the cAMP/PKA, PKC, and K<sup>+</sup>ATP channels, were tested. The cAMP/PKA pathway is the major downstream mediator of CGRP in many cell types (19). However, Rp-cAMPs (10–100 μM), a specific cell permeable inhibitor of PKA, failed to block the cell protection or the increased HSP70 expression by CGRP. A nonspecific CAMP/PKA inhibitor, H89 (10–100 nM), partially reversed the effects of CGRP (see supplemental Figs. 2 and 3). These data indicate that the cAMP/PKA pathway may not play a major role in cell protection and induction of HSP70 by CGRP in A549 cells.

Next, we tested the PKC and K<sup>+</sup>ATP channel, especially the mitochondrial K<sup>+</sup>ATP channel, because they are well known to mediate the effect of PC. Calphostin C (500 nM), a nonselective PKC inhibitor, significantly reversed the effects of CGRP on cell protection and HSP70 expression (Fig. 6, A and C). The pathophysiological role of CGRP in many cell types (19). However, a specific cell permeable inhibitor of PKA, failed to block the cell protection or the increased HSP70 expression by CGRP. A nonspecific CAMP/PKA inhibitor, H89 (10–100 nM), partially reversed the effects of CGRP (see supplemental Figs. 2 and 3). These data indicate that the cAMP/PKA pathway may not play a major role in cell protection and induction of HSP70 by CGRP in A549 cells.

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nonselective K\textsuperscript{+\textsubscript{ATP}} channel inhibitor glybenclamide (100 μM) was unable to block the effects of CGRP, whereas a selective mitochondrial K\textsuperscript{+\textsubscript{ATP}} channel blocker, 5-hydroxydecanoic acid (5HD, 10–100 μM), antagonized the CGRP-induced cell protection and HSP70 expression (Fig. 6, B and D). No further effect was detected when calphostin C (500 nM) and 5HD (100 μM) were added together (Fig. 6, B and D). These data suggest that the PKC and mitochondrial K\textsuperscript{+\textsubscript{ATP}} channels are essential in mediating the effects of CGRP on cell protection and HSP70 expression. Because PKC can activate the mitochondrial K\textsuperscript{+\textsubscript{ATP}} channel (20, 21) and blockade of both factors exerted no additive effects, they may act sequentially rather than in parallel.

Role of PKCe in CGRP Effects—Among different PKC isoforms, PKCe is regarded as a crucial mediator of the PC-induced cell protection (22, 23). Upon activation, PKCe translocates to a unique subcellular locale next to its specific substrates, including the mitochondrial K\textsuperscript{+\textsubscript{ATP}} channel (24). Indeed, we have detected the CGRP-induced membrane translocation of PKCe in A549 cells. This translocation began at 10 min and gradually increased up to 1 h after CGRP (100 nM) incubation (Fig. 7). After 1 h of CGRP treatment, the ratio of cytosolic fraction to membrane fraction of PKCe was reversed because of the significant translocation of PKCe to the membrane. Therefore, CGRP promotes the activation and translocation of PKCe in A549 cells.

The question that followed was whether the activation and translocation of PKCe play roles in CGRP-induced cell protection and HSP70 expression. To address this issue, we introduced a specific and cell-permeable PKCe inhibitory peptide (PKCV1–2, 10 μM) into A549 cells prior to CGRP treatment. Preincubation with PKCV1–2 totally abolished the effect of CGRP on cell protection (Fig. 8A) and HSP70 expression (Fig. 8B). These data strongly demonstrate that PKCe is the intracellular mediator of CGRP-induced cell protection and HSP70 expression in H/R of A549 cells.

**DISCUSSION**

In the present study, we have demonstrated that CGRP mediates the protective effect of hypoxic preconditioning against H/R injury in human alveolar epithelial cells through the activation of PKCe and expression of HSP70. Preconditioning promotes the secretion of CGRP from A549 cells, and CGRP provides feedback in an autocrine manner, interacting with its receptors on the cell membrane. Among various intracellular signaling pathways of CGRP, the activation and translocation of PKCe (but not the cAMP/PKA pathway) plays critical roles in the recruitment of downstream factors, including mitochondrial K\textsuperscript{+\textsubscript{ATP}} channels, to finally induce HSP70 expression, thus protecting against H/R injury. Therefore, we show, for the first time, that PKCe is the intracellular mediator of CGRP-induced cell protection and HSP70 expression in H/R of A549 cells.
The mediators of PKC and HSP70 expression after H/R (4).

The present study provides a unique example of how a locally released bioactive agent that plays important regulatory roles under physiological conditions is also required for dealing with pathological circumstances. Studying the trigger phase of ischemic PC has implications for pharmacological PC, which involves the use of certain substances to mimic the effect of ischemic PC. So far, most of the substances used are metabolites or by-products of ischemia and reperfusion. For example, adenosine, in which the cell protective role and detailed intracellular mechanisms are well explored, is the metabolite of nucleoside accumulated during ischemia (25). Reactive oxygen species not only trigger but also mediate PC and are mainly produced during reoxygenation by mitochondria (26). One of the agents comparable with CGRP is nitric oxide, an important regulatory of normal arterial tension, which may also protect against H/R injury (27). However, the role of nitric oxide is controversial, because it shows protective as well as detrimental effects depending on different mechanisms (28).

In the case of CGRP, studies by us and others support the diverse beneficial effects of CGRP under physiological and pathological conditions. Recently, we have shown that CGRP secretion can be induced by an inflammatory factor in type II alveolar epithelial cells, and the released CGRP plays a negative feedback role by suppressing the expression of chemokines in these cells (14). Here, we have shown that CGRP is also secreted from alveolar epithelial cells in response to PC, and the CGRP receptor blockade abolishes the effect of PC in these cells (Fig. 2). Therefore, the lung may be a beneficiary of CGRP protection in both inflammation and H/R injury. Indeed, as we manipulated CGRP expression by siRNA, a novel approach that can effectively lower messenger RNA stability and translation without influencing the genome profile (29), the decreased CGRP expression and secretion correlates well with the increased cell damage and decreased HSP70 expression (Figs. 3A and 5B). However, overexpression of the CGRP gene by stable transfer of CGRP cDNA protects the cell as effectively as hypoxic PC (Fig. 3B). These two lines of evidence, from different directions, reach the same conclusion that the self-secreted CGRP is both necessary and sufficient in mediating the protective effect of PC in lung cells. These results also extend the previous notion of the role of CGRP in ischemia of the heart and intestine (12, 13).

Because different stress signaling pathways converge on HSP70 and cells rely on the chaperoning property of HSP70 for maintaining their functional and structural integrity and withstanding H/R injury (30), we hypothesize that CGRP also recruits HSP70 to carry out its protective effect in A549 cells. Indeed, CGRP significantly increases HSP70 expression to a level comparable with that induced by PC. Furthermore, HSP70 expression is proportional to CGRP expression in A549 cells and is inhibited by blockers of the CGRP downstream signaling pathway (Fig. 5). Therefore, HSP70 can be the key end effector mediating CGRP-induced cell protection.

While dissecting the detailed post-receptor signaling of CGRP, we found that the well established $G_s/cAMP/PKA$ pathway that mediates most of the effects of CGRP in other cell types plays a minor, if any, role in protecting A549 cells from H/R injury. However, it has been proposed that CGRP can activate phospholipase C via $G_{q11}$ and increase intracellular Ca$^{2+}$ independent of the cAMP/PKA pathway in osteoblast cells (31, 32). It is unknown whether other signaling pathways are activated by CGRP in alveolar epithelial cells. The PKC inhibitor and mitochondrial K$^{+}_{ATP}$ blocker results strongly suggest that the PKC/mK$^{+}_{ATP}$ pathway is responsible for the effect of CGRP on HSP70 expression and cell protection in A549 cells (Fig. 6). This suggestion is logical, because PKC is the major survival pathway mediating the cell-protective effects of various agents against H/R injury in many cell types (33). The mitochondrial K$^{+}_{ATP}$ channel, which have been identified pharmacologically but not structurally, is the

![Effect of PKC on PKCe membrane translocation](image). A, representative Western blotting results showing membrane and cytosol fraction of PKCe after incubation with CGRP for the indicated times. B, summarized data. $n = 4$.

![Effect of PKC-specific inhibitory peptide (PKCV1-2) on CGRP-induced cell protection](image). A, PKC-specific inhibitory peptide (PKCV1-2, 10 $\mu $M) was added to A549 cells prior to CGRP treatment. CGRP was added 1 h before H/R. $n = 4$. $\ast$, $p < 0.05$ versus H/R. $\#$, $p < 0.05$ versus CGRP+H/R.
downstream target of PKC (20, 21, 34). As well, the expression of HSP70 is also regulated by PKC during PC (35).

To appraise the role of PKC in mediating the protective effect of CGRP against H/R injury, we focused on the ε subtype of PKC because PKCε activation protects cells against H/R injury (36). Our results indicate that PKCε is activated and translocated to the membrane fraction of the cells by CGRP (Fig. 7). Furthermore, activated PKCε mediates the protective effect of CGRP as well as HSP70 expression in A549 cells (Fig. 8). Thus, PKCε (but not the CAMP/PKA pathway) represents the downstream signaling of CGRP in alveolar epithelial cells. Whether other subtypes of PKC are also activated and involved in the effect of CGRP remains elusive. Our preliminary data allowed us to suggest that the δ subtype of PKC may not be activated by CGRP in A549 cells. Because PKCδ is thought to mediate the detrimental effect of reperfusion injury (37), the selective activation of protective PKC subtypes by CGRP merits investigation to avoid unnecessary side effects.

Our finding of CGRP as an intrinsic factor that protects lung cells from H/R damage will not only benefit investigations into therapy for obstructive and ischemic pulmonary diseases but also provide a new candidate for treating other ischemic diseases, such as myocardial infarction and stroke. Although previous studies have shown the activation of PKC by CGRP in osteoblast cells (38), our study is the first to clarify that the PKCδ subtype is selectively activated by CGRP in alveolar epithelial cells. Further studies are warranted to elucidate whether the CGRP/PKCε/HSP70 pathway also plays a role in the effect of CGRP in other cell types, especially the endothelial cells, cardiac myocytes, and neurons. The detailed downstream mediators of CGRP and their sequential activation should be clarified, as should the possible interaction of CGRP with other important factors, such as reactive oxygen species and nitric oxide in H/R.

In summary, alveolar epithelial cells secrete CGRP in response to ischemic PC. CGRP mediates the protective effect of PC in these cells in an autocrine manner. The activation of PKCδ, which leads to the opening of the mitochondrial K⁺/ATP channel and induction of HSP70 expression, is the major downstream signaling effect of CGRP against H/R injury.

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J. Biol. Chem. 2005, 280:20325-20330.
doi: 10.1074/jbc.M413864200 originally published online March 22, 2005

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