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Original Contribution

Involvement of Na^+/K^+-ATPase in hydrogen peroxide-induced hypertrophy in cardiac myocytes

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Abstract

We have shown that increased production of reactive oxygen species (ROS) was required for ouabain-induced hypertrophy in cultured cardiac myocytes. In the present study we assessed whether long-term exposure of myocytes to nontoxic ROS stress alone is sufficient to induce hypertrophy. A moderate amount of H_2O_2 was continuously generated in culture media by glucose oxidase. This resulted in a steady increase in intracellular ROS in cultured cardiac myocytes for at least 12 h. Such sustained, but not transient, increase in intracellular ROS at a level comparable to that induced by ouabain was sufficient to stimulate protein synthesis, increase cell size, and change the expression of several hypertrophic marker genes. Like ouabain, glucose oxidase increased intracellular Ca^{2+} and activated extracellular signal-regulated kinases 1 and 2 (ERK1/2). These effects of glucose oxidase were additive to ouabain-induced cellular changes. Furthermore, glucose oxidase stimulated endocytosis of the plasma membrane Na^+/K^+-ATPase, resulting in significant inhibition of sodium pump activity. While inhibition of ERK1/2 abolished glucose oxidase-induced increases in protein synthesis, chelating intracellular Ca^{2+} by BAPTA-AM showed no effect. These results, taken together with our prior observations, suggest that ROS may cross talk with Na^+/K^+-ATPase, leading to the activation of hypertrophic pathways in cardiac myocytes.
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Keywords: ROS; Na^+/K^+-ATPase; Ouabain; Cardiac myocytes; Hypertrophy

Introduction

Na^+/K^+-ATPase, or sodium pump, is a ubiquitous transmembrane enzyme that has long been ascribed the function of transporting Na^+ and K^+ across the plasma membrane [1,2]. Recently, we have demonstrated that Na/K-ATPase is an important signal transducer in cardiac myocytes [3–6]. Binding of ouabain to the Na^+/K^+-ATPase stimulates multiple pathways including activation of phospholipase C (PLC)/protein kinase C (PKC) cascade [7] and Src/EGFR/Ras/Raf/ERK1/2 cascade [8] and increased production of ROS [9]. ROS, such as the superoxide radical and H_2O_2, are continuously produced in most cells, and their levels are regulated by a number of enzymes and physiological antioxidants. It has been known that excessive generation of ROS is associated with cell injury in a variety of cardiovascular disorders [10–13], including atherosclerosis, ischemic heart disease, hypertension, and cardiac failure. Recently, it has become clear that ROS also act as an important second messenger in several signal pathways that control gene transcription [14,15] and cell growth [10,16–19]. ROS generation is linked to the pathways of many hypertrophic stimuli including the Na^+/K^+-ATPase ligands [9,20,21], agonists for G_q/G_{11}-coupled receptors [22–24], growth factors [25,26], and mechanotransduction [27].

We observed that partial inhibition of Na^+/K^+-ATPase by ouabain increased ROS production in cardiac myocytes.

Abbreviations: CM-DCFH, chloromethyl dihydrodichlorofluorescein diacetate; ERK, extracellular signal-regulated kinase; NAC, *N*-acetylcysteine; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Addition of antioxidant *N*-acetylcysteine (NAC) abolished ouabain-induced protein synthesis and expression of skACT and ANF as well as ouabain-induced down-regulation of the Na^+/K^+-ATPase α_3 gene [9]. These data, together with other reports [26–28], indicate that ROS are involved in the activation of hypertrophic pathways. However, whether increases in ROS stress alone are sufficient to induce hypertrophic growth remains to be established. In this study, we developed a chronic ROS stress model, assessed whether a sustained and moderate increase in intracellular ROS alone can stimulate hypertrophic growth, and explored the molecular mechanism of this ROS-induced cell growth in cultured neonatal rat cardiac myocytes.

Experimental procedures

Materials

TRI reagent for RNA isolation was purchased from Molecular Research Center, Inc. (Cincinnati, OH). Radionucleotides (^{32}P labeled, about 3000 Ci/mmol) and $^{86}Rb^+$ were obtained from Dupont NEN (Boston, MA). [^{3}H]Phenylalanine was purchased from Amersham (Cleveland, OH). Fura-2 AM and chloromethyl dihydrodichlorofluorescein diacetate (CM-DCFH) were obtained from Molecular Probes (Eugene, OR). The antibodies used and their sources were as follows: the polyclonal anti-ERK1/2 antibody, the monoclonal anti-phospho-ERK1/2 antibody, the goat anti-rabbit, and the goat anti-mouse-conjugated HRP secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminescence ECL kit was purchased form Pierce (Rockford, IL). The Optitran nitrocellulose membranes were obtained from Schleicher and Schuell (Keene, NH). All research on rats was done according to procedures and guidelines approved by the Institutional Animal Care and Use Committee.

Cell preparation and culture

Cultures of neonatal rat cardiac myocytes were prepared as described in our previous work [5]. Briefly, myocytes were isolated from ventricles of 1-to 2-day-old Sprague-Dawley rats and purified by centrifugation on Percoll gradients. Myocytes were seeded at a density of 1×10^5cells/cm^2 and cultured in a medium containing 4 parts of DMEM and 1 part of Medium 199, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum for 24 h, and then incubated in serum-free medium for 48 h before use for the experiments. These cultures contain more than 95% myocytes as assessed by immunofluorescence staining with a myosin heavy chain antibody.

Fluorescence microscopic measurements of intracellular Ca^{2+} concentration and ROS production

Myocytes were cultured on laminin-coated glass coverslips in 6-well plates. Intracellular Ca^{2+} was measured by fura-2 AM and intracellular ROS were assessed with the ROS-sensitive fluorophore CM-DCFH as described previously [9]. Briefly, fura-2 AM fluorescence was recorded using an Attofluor imaging system (Atto Instruments) at excitation wavelength of 340/380 nm and at emission wavelength of 505 nm. Under each experimental condition time-averaged signals were obtained from about 40 single cells. Relative Ca^{2+} concentration was calculated based on the fluorescence ratio and Ca^{2+} calibration curve. To measure intracellular ROS production, cells were loaded with 10 μM CM-DCFH diacetate for 15 min at room temperature in the dark. The coverslip was affixed to a culture chamber and perfused with the same culture medium without phenol red. Under each experimental condition about 15 single myocytes were imaged with an Attofluor imaging system (Atto Instruments), and CM-DCF fluorescence was measured at excitation wavelength of 480 nm and emission wavelength of 520 nm.

Protein synthesis

Protein synthesis in cultured myocytes was assayed by [^{3}H] phenylalanine incorporation as we described before [4,9]. Cells were cultured in 12-well plates, treated with glucose oxidase and other agents as indicated for 44 h, and continued for an additional 4 h in the presence of 0.5 μCi of [^{3}H]phenylalanine/well before total protein and [^{3}H]phenylalanine incorporation were assayed.

Northern blot

Total RNA was isolated using TRI reagent according to the manufacturer's protocol. Northern blot was done as previously described [9]. Routinely, about 20 μg of total RNA was subjected to gel electrophoresis, transferred to a Nytran membrane, UV-immobilized, and hybridized to ^{32}P-labeled probes. Autoradiograms obtained at −70°C were scanned with a Bio-Rad densitometer. The relative amount of RNA in each sample was normalized by using GAPDH mRNA as an internal control.

Assay for $^{86}Rb^+$ uptake by cardiac myocytes

The initial rate of Rb^+ uptake through the Na^+/K^+-ATPase was measured in the presence or absence of 1 mM ouabain as we described before [9]. Monensin (20 μM) was added to the medium for 10 min at 37°C prior to the initiation of the $^{86}Rb^+$ uptake assay to assure that the maximal capacity of active uptake was measured.

Measurement of ERK1/2 activity

Activation of ERK1/2 in cultured myocytes was determined as we previously reported [29] by Western blot using a monoclonal anti-phospho-ERK1/2 antibody that detects ERK1/2 only when they are phosphorylated at Thr202 and Tyr204. The same blots were stripped and probed with a polyclonal antibody recognizing the total amount of ERK1/2 to account for equal loading. The blots were scanned with a Bio-

Rad densitometer to quantify phospho-and total ERK1/2 signals.

Labeling of cell surface Na/K-ATPase by biotinylation

Cell surface protein biotinylation was performed as described before [30]. Proteins bound to the ImmunoPure immobilized streptavidin-agarose beads were eluted and then resolved on SDS-PAGE followed by immunoblotting.

Confocal microscopy

Cell growth, immunostaining, and confocal microscopy were performed as described before with little modification [6]. Briefly, after treatment, myocytes were fixed with 2% paraformaldehyde for 10 min at room temperature and then permeabilized in 0.3% Triton X-100 for 10 min. Cells were blocked by Image-iT FX signal enhancer (Molecular Probes) for 30 min. For cell size and actin staining, cells were incubated with Alexa 488-conjugated Phalloidin (Molecular Probes) for 2 h at room temperature. For Na/K-ATPase endocytosis, cells were incubated with anti-Na/K-ATPase-α_1 monoclonal antibody (Upstate Biotechnology, 1:50) and followed by incubation with Alexa 488-conjugated secondary antibodies. The coverslips were mounted with ProLong Gold antifade reagent (Molecular Probes). Confocal image was captured by a Leica TCS SP2 spectral confocal scanner and a Leica DMIRE2 microscope (Leica, Mannheim, Germany) equipped with a 63× oil immersion objective. Visualization and analysis were performed using Leica confocal microscope system software.

Statistics

Data are given as the mean ± SE. Statistical analysis was performed using the Student's t test, and significance was accepted at $p < 0.05$. Each presented immunoblot is representative of the similar results of at least three separate experiments.

Results

Addition of glucose oxidase generates chronic ROS stress in cardiac myocytes

Since increases in ROS play a pivotal role in ouabain-induced hypertrophic growth, it is of interest to extend our studies on ouabain and determine if chronic exposure of cardiac myocytes to elevated ROS regulates cardiac growth and gene expression in a pattern similar to that of ouabain. In order to achieve this goal, we added glucose oxidase to the culture medium to continuously generate H_2O_2. It is known that glucose oxidase produces H_2O_2 in the presence of glucose [31]. As expected, addition of this enzyme to the culture medium caused a sustained rise in intracellular ROS (Fig. 1A). Addition of catalase (50 U/ml) completely blocked glucose oxidase-induced increases in intracellular ROS, indicating that increases in intracellular ROS were due to glucose oxidase-catalyzed

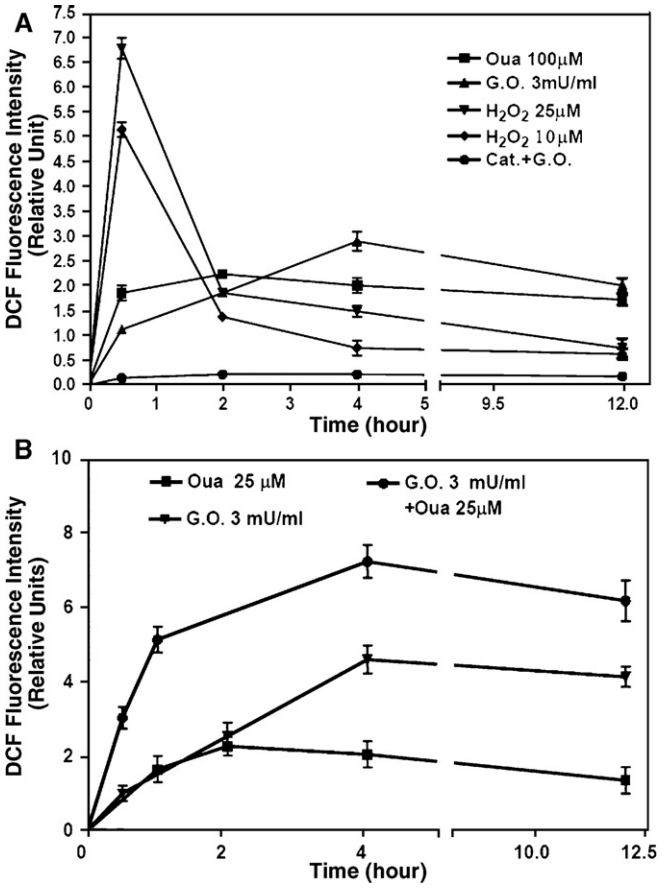

Fig. 1. Stimuli-induced changes in intracellular ROS. (A) Cardiac myocytes were treated with indicated concentrations of glucose oxidase (G.O.), H_2O_2, ouabain (Oua), or catalase (50 U/ml) plus glucose oxidase (Cat.+G.O.) for various times. (B) Cardiac myocytes were treated with glucose oxidase and/or ouabain for various times. The stimuli-treated cells were then loaded with 10 μM CM-DCFH diacetate and assayed for the fluorescence intensities of the oxidized probe as described under Experimental procedures. The value of every time point was calibrated by the value of the control cells. Each value is the mean± SE of determinations of 15 single cells in four independent experiments.

H_2O_2 production. The effects of glucose oxidase on intracellular ROS levels were dose and time dependent. Glucose oxidase-induced increases in ROS reached maximal levels after 4 h and lasted for at least 12 h. In comparison, exposure of myocytes to a bolus 10–25 μM H_2O_2 caused a much faster increase in intracellular ROS. However, intracellular ROS decreased significantly from the peak after 1 h H_2O_2 exposure and approached the control level after 2–4 h of incubation (Fig. 1A). When compared to added bolus H_2O_2, 100 μM ouabain increased intracellular ROS to levels similar to that induced by 2 μM H_2O_2, while 3 mU/ml glucose oxidase raised intracellular ROS to that produced by 5 μM H_2O_2 (data not shown). Clearly, like ouabain, the addition of glucose oxidase (but not a bolus H_2O_2) to culture medium could cause a sustained increase in intracellular ROS levels. Moreover, the treatment with ouabain and glucose oxidase simultaneously caused an additive effect on ROS generation (Fig. 1B). Therefore, we used this glucose oxidase model in the following studies to investigate whether exposure of cultured cardiac myocytes to chronic ROS stress induced hypertrophic growth.

Chronic ROS stress stimulates hypertrophic growth in cardiac myocytes

To address whether an increase in ROS alone is sufficient to cause hypertrophic growth in cardiac myocytes, we first measured the effects of glucose oxidase on the expression of hypertrophic marker genes using Northern blot analysis. A characteristic feature of hypertrophic growth in adult cardiac muscle is the activation of a so-called "fetal program" that consists of the recapitulation of a pattern of cardiac-specific gene expression of the prenatal heart. For example, the rodent skACT is expressed in the fetal ventricle, and then replaced by the adult isoform, cardiac α-actin, during postnatal development. Many hypertrophic stimuli including ouabain increase the expression of skACT as well as ANF in cultured myocytes [32]. In addition, these stimuli also reduce the expression of the α3 subunit of the Na^+/K^+-ATPase [9]. As depicted in Figs. 2A and 2B, glucose oxidase, like ouabain, caused a dose-dependent increase in the expression of both skACT and ANF mRNA. In addition, it also reduced the expression of the Na^+/K^+-ATPase α_3 mRNA. The glucose oxidase-induced decrease in α_3 mRNA and increases in skACT and ANF mRNA were completely blocked by either catalase or NAC. To further test whether increases in ROS alone are sufficient to induce hypertrophic growth, cells were exposed to different concentrations of glucose oxidase and assayed for morphological changes and [^{3}H]phenylalanine incorporation. As shown in Fig. 3, glucose oxidase clearly increased the cell size and stimulated actin reorganization in the cultured myocytes as other hypertrophic stimuli [26,33]. Consistently, glucose oxidase also stimulated protein synthesis in a dose-dependent manner (Fig. 4). It is important to note that under the same experimental conditions, a bolus H_2O_2 at 10 μM or lower showed no effect on protein synthesis (data not shown), while a bolus 25 μM H_2O_2 caused a significant decrease in total protein content. When cell viability was determined, 10 μM H_2O_2 had no effect, while 25 μM H_2O_2 caused a statistically significant loss (about 15%) of cell viability after 24 h incubation. On the other hand, ouabain up to 100 μM and glucose oxidase up to 3 mU/ml showed no effect on cell viability after 48 h of exposure. These findings, taken together with those in Fig. 1, clearly showed potential pitfalls associated with use of a high concentration of bolus H_2O_2 in studying long-term effects of ROS signaling. A low dose of H_2O_2, as shown in Fig. 1A, would be destroyed quickly by the cell antioxidant system so that it could not exhibit a significant effect on cell growth whereas a high dose might cause cell toxicity. In short, the above data indicate that increases in intracellular ROS alone were sufficient to stimulate hypertrophic growth in cultured neonatal rat cardiac myocytes.

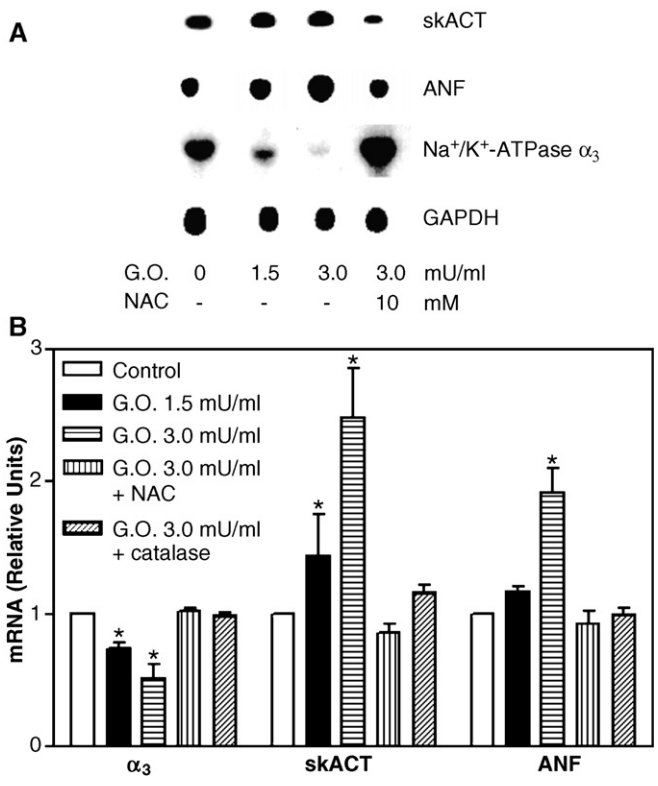

Fig. 2. Effects of glucose oxidase on the expression of hypertrophic marker genes. Myocytes were treated with different concentrations of glucose oxidase (G.O.) alone or with 10 mM NAC or 50 U/ml catalase for 12 h, and total RNA was isolated and assayed for cardiac gene expression using Northern blot. Panel A show a representative Northern blot. Lane 1, control; Lane 2, 1.5 mU/ml glucose oxidase; Lane 3, 3.0 mU/ml glucose oxidase; Lane 4, 3.0 mU/ml glucose oxidase plus NAC. Panel B shows quantitative data. Values were normalized to those of corresponding GAPDH measured on the same blot and expressed relative to control value of one. Each value is the mean ± SE of three experiments.

ROS-induced cardiac hypertrophy shared signal pathways similar to those activated by ouabain binding to the Na^+/K^+-ATPase

The Na^+/K^+-ATPase serves as an important signal transducer. Binding of ouabain to the Na/K-ATPase activates multiple pathways including increases in ROS [9], intracellular Ca^{2+} [3], and activation of ERK1/2 [7,34]. There is evidence that increases in intracellular Ca^{2+} and subsequent activation of the calcineurin/NF-AT pathway as well as the stimulation of ERK1/2 play an important role in ouabain and other stimuli-induced hypertrophic growth [4,35]. Because glucose oxidase and ouabain caused a similar pattern of changes in gene expression and cell growth, the following experiments were performed to explore whether glucose oxidase affected intracellular Ca^{2+} and ERK1/2 activity in cardiac myocytes. Activation of ERK1/2 was measured by Western blot using an anti-phospho-ERK antibody. As depicted in Fig. 5, addition of 3 mU/ml glucose oxidase increased phosphorylation levels of ERK1 (upper 44-kDa band) and 2 (42-kDa band) in a time-dependent manner. This time course of ERK activation correlated with the rise in intracellular ROS (Fig. 1A). When intracellular Ca^{2+} was measured in fura-2-loaded myocytes, glucose oxidase caused a gradual rise in intracellular Ca^{2+} as shown in Fig. 6. The maximal effects were observed after the cells were exposed to glucose oxidase for 2–4 h and the intracellular Ca^{2+} remained elevated for at least 12 h.

To investigate the functionality of the ERK1/2 pathway in glucose oxidase-induced hypertrophic growth, myocytes were

Fig. 3. Effects of glucose oxidase on sarcomere reorganization and cell size. Myocytes were treated with glucose oxidase or/and ouabain for 24 h, fixed, and then stained with Alexa 488-conjugated phalloidin as described under Experimental procedures. Scale bar = 10 μm. A representative experiment is shown and the same experiments were repeated three times.

pretreated with a MEK inhibitor (PD98059) for 30 min and then exposed to glucose oxidase. As shown in Fig. 7, PD98059 completely abolished the effect of glucose oxidase on protein synthesis. To test the role of a rise in intracellular Ca^{2+} and the resulting activation of the calcineurin/NF-AT pathway [36–38], myocytes were preloaded with 10 μM BAPTA-AM and then exposed to glucose oxidase. Although BAPTA significantly decreased basal protein synthesis, it did not block glucose oxidase-induced protein synthesis (Fig. 8). Because ouabain signals via the activation of Src [39] and increases in ROS are known to stimulate Src [40,41], we also tested the role of Src family kinase. As depicted in Fig. 7, inhibition of Src by PP2 completely blocked glucose oxidase-induced increases in protein synthesis.

Cross talk between Na^+/K^+-ATPase and ROS

We have shown that partial inhibition of Na^+/K^+-ATPase by ouabain increased ROS production in cardiac myocytes [9]. Since our previous work showed that ROS had the potential to interact with the cardiac Na^+/K^+-ATPase [35], we reasoned that the Na^+/K^+-ATPase could serve as a target for ROS to transmit the signals to downstream effectors, thus contributing to ROS-induced changes in gene expression and cardiac hypertrophy in cultured neonatal cardiac myocytes. To address this issue, we first determined whether ouabain and ROS have additive effects on cell signaling, growth, and gene expression. Consistent with the findings illustrated in Fig. 1B, ouabain potentiated the effects of glucose oxidase on gene expression (data not shown) and protein synthesis (Fig. 4), supporting the notion that glucose and ouabain share similar pathways of cardiac hypertrophy.

To further test whether ROS cross talk with the ouabain-activated pathways, we measured the effects of ROS on the cardiac Na^+/K^+-ATPase activity. It showed that sustained increases in ROS stress produced by glucose oxidase could cause a time-dependent inhibition of Na^+/K^+-ATPase (Fig. 9). Inhibition of the Na^+/K^+-ATPase by glucose oxidase correlated well with the increases in intracellular ROS concentration. The maximal inhibition (about 40%) occurred after 4 h of glucose oxidase exposure. Since the enzyme is significantly inhibited by

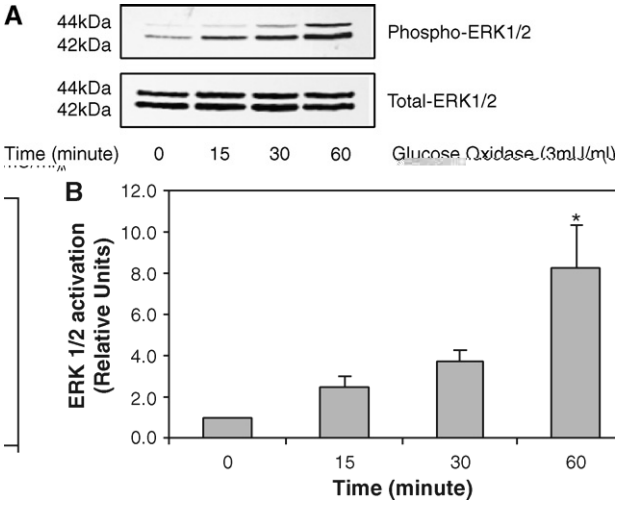

Fig. 5. Effect of glucose oxidase on activation of ERK1/2. Myocytes were exposed to glucose oxidase (3 mU/ml) for the indicated times and assayed for ERK1/2 activation as described under Experimental procedures. (A) Representative immunoblots. Membranes were detected with anti-phosphorylated ERK1/2 antibody and then stripped and reprobed by an anti-ERK1/2 pan antibody that recognizes total ERK1/2. (B) Quantitative data from three independent experiments. The phosphorylated ERK1/2 values were normalized against the total ERK1/2 signal. This ratio was then expressed relative to control value of one. Values are means ± SE *$p < 0.05$ vs control.

Fig. 4. Effects of glucose oxidase and ouabain on protein synthesis. Myocytes were treated with glucose oxidase (G.O.) or/and ouabain (Oua) for 48 h and then assayed for [^{3}H]phenylalanine incorporation as indicated under Experimental procedures. Values are means ± SE of four experiments. *$p < 0.05$, ** $p < 0.01$ compared to control.