Heat Shock Protein 90β1 Is Essential for Polyunsaturated Fatty Acid-induced Mitochondrial Ca\(^{2+}\) Efflux

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Nonesterified fatty acids may influence mitochondrial function by alterations in gene expression, metabolism, and/or mitochondrial Ca\(^{2+}\) homeostasis. We have previously reported that polyunsaturated fatty acids induce Ca\(^{2+}\) efflux from mitochondria, an action that may deplete [Ca\(^{2+}\)]\(_{\text{intr}}\) and thus contribute to nonesterified fatty acid-responsive mitochondrial dysfunction. Here we show that the chaperone protein heat shock protein 90 β1 (hsp90β1) is required for polyunsaturated fatty acid-induced mitochondrial Ca\(^{2+}\) efflux (PIMCE). Retinoic acid induced differentiation of human teratocarcinoma NT2 cells in association with attenuation of PIMCE. Proteomic analysis of mitochondrial proteins revealed that hsp90β1, among other proteins, was reduced in retinoic acid-differentiated cells. Blockade of PIMCE in NT2 cells by 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin, a known inhibitor of the chaperone activity of hsp90, and hsp90β1 RNA interference demonstrated that hsp90β1 is essential for PIMCE. We also show localization of hsp90β1 in mitochondria by Western blot and immunofluorescence. Distinctive effects of inhibitors binding to the N or C terminus of hsp90 on PIMCE in isolated mitochondria suggested that the C terminus of hsp90β1 plays a critical role in PIMCE.

Defective mitochondrial function has been observed in type 2 diabetes and is proposed to be a major contributing factor in the pathogenesis and progression of the disease (1, 2). Although the mechanism leading to mitochondrial dysfunction in diabetes remains under intensive investigation, a critical role for nonesterified fatty acids (NEFA)\(^2\) and/or fatty acid metabolites is emphasized by an increasing body of evidence (3–5). NEFA may influence mitochondrial function by alterations in gene expression (6), metabolism (7), and/or mitochondrial Ca\(^{2+}\) homeostasis (8, 9). Ionic Ca\(^{2+}\) in the mitochondria regulates substrate oxidation by activation of mitochondrial dehydrogenases (9). Deficiency of pyruvate dehydrogenase (PDH) activity in pancreatic islet β-cells (10), cardiomyocytes (11), and skeletal muscles (7, 12, 13) has been demonstrated in diabetes. The degree to which PDH is dephosphorylated (i.e. the balance between phosphorylation and dephosphorylation) determines the level of enzyme activity. The concentration of mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{intr}}\)) is an important activator of PDH phosphatase, which dephosphorylates and activates PDH. Additionally, [Ca\(^{2+}\)]\(_{\text{intr}}\) has been shown to activate at least three other mitochondrial dehydrogenases (i.e. glycerol 3-phosphate dehydrogenase, NAD-linked isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase), all of which play important roles in substrate oxidation (9). NEFA, which are chronically elevated in type 2 diabetes, inhibit PDH by mechanisms thought to involve FA oxidation and elevation of acetyl-CoA. We have previously reported that polyunsaturated fatty acids (PUFA) induce Ca\(^{2+}\) efflux from mitochondria (8), an action that could deplete [Ca\(^{2+}\)]\(_{\text{intr}}\) and thus contribute to NEFA-responsive reduction of PDH activity and mitochondrial dysfunction. The PUFA-induced mitochondrial Ca\(^{2+}\) efflux (PIMCE) cannot be blocked with cyclosporine A and bongkrekic acid, two inhibitors of the mitochondrial permeability transition pore. However, the pathway(s) underlying PIMCE has not previously been defined.

In the current work, we have used proteomic analysis to demonstrate a parallel reduction in PIMCE and the level of heat shock protein 90β1 (hsp90β1) in NT2 cells treated with retinoic acid (RA). Further studies with pharmacological inhibitors and RNA interference (RNAi) indicate that hsp90β1 plays an essential role in PIMCE in NT2 cells.

EXPERIMENTAL PROCEDURES

Materials—Fura-2 AM, Fluo-3 AM, Mitotracker Orange, and X-rhod-1 AM, as well as Dulbecco’s modified Eagle’s medium, Opti-MEM, and phosphate-buffered saline (PBS, containing 1 mM KH\(_2\)PO\(_4\), 3 mM Na\(_2\)HPO\(_4\), 154 mM NaCl, pH 7.2) powders were purchased from Invitrogen. Linoleic acid and other PUFA were from Cayman Chemical Co. (Ann Arbor, MI). The human teratocarcinoma cell line NT2 was purchased from American Type Culture Collection (Manassas, VA). Geldanamycin and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) were purchased from Invivo-
Gen (San Diego, CA). Retinoic acid, carbochol, EGTA, EDTA, and other chemicals were from Sigma.

**Cell Culture**—The NT2 cell line was cultured in 100-mm dishes in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum (FBS) and penicillin (50 units/ml)/streptomycin (50 μg/ml) at 37 °C in a humidified 5% CO₂ atmosphere incubator. In some experiments, cells were treated with retinoic acid or other chemicals as indicated for various periods. Cells grown to near confluence (90%) were harvested with trypsin (0.05%)/EDTA (0.02%), and suspensions of NT2 cells were used for measurement of intracellular Ca²⁺ ([Ca²⁺]i) and preparation of mitochondria.

**Induction of NT2 Cell Differentiation**—NT2 cells were incubated with 10 μM RA for 8 weeks to induce differentiation of neuron-like cells as previously described (14).

**Measurement of [Ca²⁺]i**—NT2 cells or RA-differentiated cells suspended in HEPES/sodium/glucose (HNG) buffer containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1.2 mM KH₂PO₄, 10 mM glucose, 10 mM HEPES, pH 7.4, were labeled with fura-2 AM (1 μM) at 37 °C for 30 min with agitation at 50 rpm. Loaded cells were washed and resuspended in HNG, and [Ca²⁺]i mobilization in populations of NT2 cells or RA-differentiated cells was measured in a fluorometer (QM-6; Photon Technology International), using a cuvette with the temperature stabilized at 37 °C through a connected water bath. The ratio of fluorescence excited at 340 and 380 nm with emission at 510 nm was recorded and used to index the change of [Ca²⁺]i as previously reported (15).

**Measurement of [Ca²⁺]i**—mobilization in single NT2 cells was conducted using a Bio-Rad laser scan confocal imaging analysis system. The cells were grown in 12-well plates at a density of 10⁴ cells/well for 48 h and treated with 17-DMAG or hsp90β1 RNAi as indicated for an additional 48–72 h. Fluorescent images of individual cells were then acquired, and [Ca²⁺]i was measured as previously described (16).

**Preparation of Mitochondria and Measurement of Mitochondrial Ca²⁺ Efflux**—The preparation of mitochondria from NT2 and RA-differentiated cells and rat tissues (liver, brain, spleen, heart, kidney, and skeletal muscle from 3–4-month-old male Sprague-Dawley rats, provided by Dr. Kong Zhang, Department of Medicine, University of Texas Health Science Center, San Antonio, TX) and the measurement of PIMCE were performed as described previously with modification (8). Rat tissues were cleaned and minced in 2 ml of ice-cold mitochondrial preparation buffer (MB1) containing 250 mM mannitol, 75 mM succinic acid, 0.1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 7.4. Cleaned tissue samples were homogenized in 15–20 ml of MB1 on ice, using a Teflon-glass homogenizer (Glas-Col, Terre Haute, IN) at 50 rpm for 40 strokes. The homogenates were centrifuged at 130 × g at 4 °C for 15 min, and the supernatant was carefully removed and centrifuged at 10,000 × g to precipitate the mitochondria. The samples were resuspended and washed in MB1, and protein concentrations were determined as described (17) using bovine serum albumin as the standard and following the manufacturer’s instructions (Pierce). Mitochondrial preparations containing equivalent protein concentrations were then resuspended in 200 μl of MB1, diluted with 1.8 ml of HEPES/potassium/glucose buffer (HKG) containing 20 mM NaCl, 100 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 20 mM HEPES, pH 7.4, and loaded with 10 μM X-rhod-1 AM for 30 min at 37 °C with agitation at 20 rpm. The loaded mitochondria were resuspended in 100–150 μl of HKG and used to measure PIMCE as described in Ref. 8. Briefly, X-rhod-1 and Ca²⁺-loaded mitochondria in HKG were diluted to 2 ml of PBS0Ca (PBS supplemented with 100 μM EGTA and 1 mM MgCl₂) in a cuvette with constant magnetic stirring. The fluorescence excited at 578 nm and emitted at 602 nm was measured in a fluorometer manufactured by Photon Technology International (Lawrenceville, NJ). The base line was established by incubation of mitochondria in the cuvette without the addition of PUFA. In experiments measuring PIMCE in rat tissue mitochondria, 40 μg/ml mitochondrial protein was used for all tissues; 15–50 μg/ml mitochondrial proteins was used for measuring PIMCE in mitochondria from cultured cells. Equivalent results were obtained when linoleic acid (LA)-induced Ca²⁺ efflux from mitochondria of NT2 cells was measured in PBS0Ca or Ca²⁺-free HKG solution (containing 100 μM EGTA instead of 1 mM CaCl₂).

**Two-dimensional Gel Electrophoresis**—Isoelectrofocusing of mitochondrial proteins from NT2 and RA-differentiated neural cells was performed in a Bio-Rad PROTEAN IEF Cell with 7-cm Immobilized pH gradient strips (pH 3–10) according to the manufacturer’s instructions. The mitochondrial samples were suspended and solubilized in a buffer containing 9 mM urea, 2.5 M thiourea, 2% CHAPS, 0.8% Bio-Lyte (pH 3–10), and 15 mM dithiothreitol at room temperature for 60 min. The dissolved samples were centrifuged at 150,000 × g for 60 min at 4–6 °C. A 200-μl aliquot of each supernatant was loaded, and the ReadyStrip IPG strip (pH 4–7; Bio-Rad) was drawn gel side down through the solution without air bubbles. Each ReadyStrip Immobilized pH gradient strip was then covered with mineral oil to prevent precipitation of the urea. Following the 12-h rehydration, the strips were electrofocused for 4 h at 10,000 V. When completed, the strips were equilibrated in 5 ml of buffer containing 6 M urea, 2% SDS, 30% glycerol, 1% dithiothreitol, 50 mM Tris, pH 8.8, for 20 min. A subsequent equilibration was performed in 5 ml of the same buffer with 2.5% iodoacetamide substituted for the dithiothreitol. Protein samples in the equilibrated strips were separated on 8% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R-250 and scanned with a high resolution scanner (GS-800 Calibrated Imaging Densitometer; Bio-Rad). The protein levels in the gel images were analyzed with the PD-Quest program (Bio-Rad). The mitochondrial proteins with a molecular mass of ≥100 kDa and expression levels largely reduced in RA-differentiated cells were subjected to further analysis with mass spectrometry.

**Protein Identification by Mass Spectrometry**—Spots of interest in the SDS-PAGE were robotically excised by means of a Proteome Works spot cutter (Bio-Rad) and digested in situ with trypsin according to standard protocols based on the initial work of Mann and co-workers (18). Briefly, protein spots were excised from the gel and destained in 50% acetonitrile, 40 mM ammonium bicarbonate, pH 7.4, prior to digestion. Gel plugs were then dehydrated in 100% acetonitrile and rehydrated with 5–10 ng/μl trypsin (Promega; modified) in 40 mM ammonium bicarbonate and incubated overnight at 37 °C. The resulting
digests were analyzed by capillary HPLC-electrospray tandem mass spectrometry on a Thermo Fisher LTQ ion trap mass spectrometer coupled to an Eksigent NanoLC micro-HPLC by means of a PicoView (New Objective) nanospray interface. Capillary on-line HPLC separation of tryptic peptides was conducted using the following conditions: column, New Objective PicoFrit, 75 μm inner diameter, packed to 11 cm with C18 adsorbent (Vydac 218MSB5); mobile phase A, 0.5% acetic acid, 0.005% trifluoroacetic acid in water; mobile phase B, 90% acetonitrile, 0.5% acetic acid, 0.005% trifluoroacetic acid in water; gradient, 2% B to 42% B in 30 min; flow rate, 0.4 μl/min. A data-dependent acquisition protocol was employed consisting of one survey scan followed by seven collision-induced dissociation spectra. The uninterpreted collision-induced dissociation spectra were searched against the NCBInr data base using Mascot (Matrix Science; 10-processor in-house license). Variable modifications considered include methionine oxidation and cysteine carbamidomethylation. A 95% confidence level threshold was used for Mascot peptide scores.

**RESULTS**

Following our previous observations of PIMCE in NT2 cells, we investigated PIMCE of various rat tissues using isolated mitochondria. Mitochondria from a number of tissues tested contained 0.1% Tween 20 and incubated with Alexa 488-labeled goat anti-mouse IgG (1:1000 dilution; Invitrogen) for 1 h at room temperature. Labeled cells were washed, and images were acquired using a Bio-Rad confocal laser scan imaging system. The excitation laser beams used were 488 and 554 nm for Alexa and Mitotracker, respectively. Fluorescent images were sequentially recorded from the same field with 515 nm band pass and 570 nm long pass filters. The levels of hsp90β1 in mitochondria were determined by the fluorescence ratio of Alexa 488 and MitoTracker from eight or nine randomly selected areas in each experiment.

**Statistics**—Data are presented as means ± S.E. Comparisons were performed using a two-tailed Student’s t test or analysis of variance. A significant difference was defined at p < 0.05.
liver, spleen, and heart) showed PIMCE (Fig. 1A; data from kidney and skeletal muscle not shown). However, mitochondria from rat brain showed diminished PUFA responses compared with mitochondria of other tissues (Fig. 1A). For example, the initial rate of LA-induced mitochondrial Ca\textsuperscript{2+} efflux in the brain was less than one-tenth of that in the liver (Fig. 1B). Based on this finding, we designed experiments to identify the protein pathway(s) responsible for PIMCE. It has been demonstrated previously that following prolonged treatment with RA, NT2 cells are induced to differentiate into mature neurons (14). It is possible that the mitochondria of neurons differentiated from NT2 cells behave similar to those of neuronal brain cells. Accordingly, we hypothesized that as in the mitochondria of brain tissue, PIMCE would be attenuated in neurons differentiated from NT2 cells. As shown in Fig. 2, in control NT2 cells, LA (3 × 10^{-5} M) induced increases of intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}; Fig. 2B), and PIMCE was observed in isolated mitochondria (Fig. 2C). After treatment of NT2 cells with RA according to the procedures described (14), we observed the differentiation of neuron-like cells (cf. differentiated cells in Fig. 2D with control cells in Fig. 2A), consistent with previous findings (14). Interestingly, LA-induced [Ca\textsuperscript{2+}], responses in RA-differentiated cells were abolished (Fig. 2E), as was LA-responsive PIMCE in mitochondria isolated from these RA-treated cells (Fig. 2F).

Analysis of mitochondrial proteins from control NT2 and RA-differentiated cells with two-dimensional gel electrophoresis revealed alterations in the expression levels of multiple proteins (cf. gel images in Fig. 3, A and B). To identify the proteins

![Figure 2](image2.png)  
**FIGURE 2.** Effects of RA-induced differentiation of NT2 cells on LA (3.0 × 10^{-5} M)-induced [Ca\textsuperscript{2+}]\textsubscript{i} mobilization and Ca\textsuperscript{2+} efflux in mitochondria and on mitochondrial proteins. The cell images of control (A) and RA-differentiated cells (D) were acquired with ×200 magnification. The traces showing [Ca\textsuperscript{2+}]\textsubscript{i} mobilization in suspensions of intact cells (B and E) and mitochondrial Ca\textsuperscript{2+} efflux (C and F) are from a typical experiment.

![Figure 3](image3.png)  
**FIGURE 3.** Effects of RA on mitochondrial proteins. A and B, images of two-dimensional gel electrophoresis of mitochondrial proteins from control and RA-differentiated NT2 cells, respectively. The arrowheads and numbers in A indicate the particular protein spots selected for mass spectroscopy analysis. Corresponding positions of the spots in RA-treated cells (B) are also labeled.
that might be Ca\textsuperscript{2+} channels or transporters involved in PIMCE, we selected three protein spots for further analysis by mass spectroscopy based on the following two criteria: 1) the protein levels were undetectable or largely reduced after RA treatment; 2) the molecular mass of the proteins should be 100 kDa (marked by arrowheads in Fig. 3A). A fourth protein spot with a molecular mass of \( \approx 30 \) kDa, which was also undetectable following RA treatment, was selected randomly as a control for mitochondrial localization. Corresponding positions of the spots in RA-treated cells are marked by the arrowheads in Fig. 3B. The three spots of interest were analyzed with mass spectroscopy and identified with Mascot in Fig. 3C). The three spots of interest were analyzed with mass spectroscopy and identified with Mascot.

A literature search for information on the functions of the above identified proteins indicated that none of the proteins of interest was known to be a mitochondrial Ca\textsuperscript{2+} channel or transporter. It has been reported that hsp90 and other chaperone proteins are involved not only in protein folding but also protein importation to the mitochondria (22, 23) and/or interactions between mitochondrial and endoplasmic reticulum Ca\textsuperscript{2+} stores (24). We thus focused on the protein hsp90\( \beta 1 \) and tested whether PIMCE in NT2 cells was affected by 17-DMAG, which binds to the N-terminal ATPase domain of hsp90 proteins and inhibits their chaperone activity. Prolonged treatment with 17-DMAG may also cause degradation of hsp90\( \beta 1 \) (37). Additionally, we also tested the effect of hsp90\( \beta 1 \) down-regulation with hsp90\( \beta 1 \) RNAi.

Treatment of cells with 17-DMAG for 48 h during culture caused a concentration-dependent inhibition of the L-A induced [Ca\textsuperscript{2+}], signal measured in suspensions of cells, with maximal effect at 10\textsuperscript{–9} to 10\textsuperscript{–8} M 17-DMAG (Fig. 4A); over the same range of concentrations, 17-DMAG had no effect on the [Ca\textsuperscript{2+}], response to the muscarinic agonist carbachol (Fig. 4B). 17-DMAG blocked the carbachol-induced [Ca\textsuperscript{2+}], signal only at high concentrations (5 \( \times 10\textsuperscript{–8} \) to 2 \( \times 10\textsuperscript{–7} \) M) that significantly reduced cell counts (Fig. 4, B and C).

Image analysis of individual NT2 cells treated with 17-DMAG (10\textsuperscript{–8} M) or hsp90\( \beta 1 \) RNAi for 48 h revealed that both treatments effectively blocked LA-induced [Ca\textsuperscript{2+}], mobi-

### Table 1

**Potential candidates of the pathway(s) responsible for PUFA-induced mitochondrial Ca\textsuperscript{2+} efflux identified by capillary-HPLC-electrospray tandem mass spectrometry**

Candidate proteins were identified in spots 1–3 from two-dimensional gel electrophoresis of mitochondrial proteins (Fig. 1G). Spot 4 was randomly selected and used as a control for localization in mitochondria.

| Spot number | Protein                                      | Database accession number | Theoretical pI/molecular mass | Sequence coverage | Mascot score |
|-------------|----------------------------------------------|---------------------------|-------------------------------|-------------------|--------------|
| 1           | Heat shock protein 90\( \beta 1 \)           | AAH66656                  | 4.76/92                       | 51                | 1521         |
| 2           | 150-kDa oxygen-regulated protein variant 1   | ABD14370                  | 5.16/111                      | 40                | 971          |
| 3           | Leucine-rich PPR motif-containing protein     | NP_573556                 | 5.81/158                      | 34                | 1192         |
| 4           | Nodal modulator 2 isoform 2                  | NP_775885                 | 5.51/134                      | 17                | 368          |
| 5           | Pre-mRNA splicing factor SF2p32              | AAA73055                  | 4.74/31                       | 19                | 330          |

### Table 2

**Sequences of matched peptides**

| Proteins                                      | Matched peptides                                                                 |
|-----------------------------------------------|----------------------------------------------------------------------------------|
| Heat shock protein 90\( \beta 1 \)            | TDDENVQVREEAAQQLDGLNASQIQF, FAQAEVNR, KNEIIFRELINASADALDK, NLLTVTPOTGAVMREELK    |
| Leucine-rich PPR motif-containing protein      | TEFARHRWDTLQKGLAVYDVSNYNLLKYYQNEVYKSPTSDFLAKMEANIQPNTVYQR, I1GFMK, AGDMENAENLTVMR, GDIDVHKQTEL, SELHMLDR, EKNVQGHEIELK, SNTLPSLQSRRSLLGFR, EEHLR, IPENYVR, DADHLVESK, AENQPRIDVLK, VDLKL, GEEVTIK, TVKEGETDLIQK, LQITQPGR, LVELTQLK, DQMYNLIK, ADAOVNQKIQENVIPR, LIAELIR, HSLLNSSASTETDPDFQK, KQAYDFLNK, GFLNDTANSLRILITQVR, EAVTTIKTQLDQQTTSRLAVTR, GDVENIEVQKMLNGLEDSIGL, SMVFINNIALQIK, KVIEQELQAVESKISAMER, EAYNLSMLSDSYKEDVTSKALYEHTLAK, LDDLFKLRYAHLK |
| Nodal modulator 2 isoform 2                   | GPQPGPLVAGQVQLSR, IQVSTVTQPQGGK, IFTDFVAPSLRDFVTEHDSLK, ISIRIFPDTVK, VVLSQSDKDK, VQVMYPVEATR, SLQGSLG, ENVGYNILSK, HHVLTGTDKMDYDVTLSKIDSQALVGLPLK, EQQLAEIAR, LIEHGG, GASSPILVFIDK, ITIITYR, AEGNDHIERALPHR, HITLIFNPR, QGVGALGQAASDNSGPDACKRK |
Hsp90β1 and Fatty Acid-induced Mitochondrial Ca^{2+} Efflux

Figure 4. Effects of 17-DMAG on LA (3.0 × 10^{-9} M) and carbachol (10^{-4} M)-induced [Ca^{2+}]_{i} mobilization in NT2 cells. LA and carbachol responses are shown in A and B, respectively; the effects of 17-DMAG on cell numbers are illustrated in C. Treatment of cells with increasing concentrations of 17-DMAG for 48 h during culture caused concentration-dependent inhibition of the LA-induced [Ca^{2+}]_{i} signal. The values presented in the figures are relative changes to control (100%) from 3–6 experiments.
with maximal inhibition observed at $2 \times 10^{-3}$ M. These data suggest a critical role for the C terminus of hsp90β1 in PIMCE.

**DISCUSSION**

In the present study, we have identified the essential role of hsp90β1 in LA-induced mitochondrial Ca$^{2+}$ efflux. The data presented in this study demonstrate an important novel function for the well-known chaperone protein hsp90β1 (i.e., its involvement in PIMCE, a process by which elevated NEFA may lead to altered mitochondrial Ca$^{2+}$ homeostasis and mitochondrial dysfunction).

NEFA in animal and human plasma and tissues are composed of saturated FA, monounsaturated FA, and PUFA (29), and there is evidence that the deleterious effects of these different types of FA may be mediated by distinct cellular pathways. For example, a recent study indicates that saturated and monounsaturated FA, but not PUFA, are effective in increasing cellular levels of diacylglycerol and thus causing stimulation of protein kinase C (30). In our study, PIMCE was observed only with PUFA and not with saturated or monounsaturated FA (8). However, the effect of PUFA on mitochondrial Ca$^{2+}$ could potentially alter the metabolism of saturated and monounsaturated FA through changes in substrate oxidation.

Although the majority of studies examining the deleterious effects of FA have been performed using saturated FA, LA is a major component of NEFA, and its concentration is significantly elevated in diabetes (29). The PIMCE response to LA and other PUFA may account for a portion of the adverse effects of NEFA, especially at the mitochondrial level, whereas saturated and monounsaturated FA may exert their effects via other pathways.

Multiple Ca$^{2+}$ transport pathways responsible for mitochondrial Ca$^{2+}$ uptake and Ca$^{2+}$ efflux have been characterized functionally. The pathways linked to mitochondria...
Mitochondrial 
Ca\(^{2+}\) uptake include the mitochondrial 
Ca\(^{2+}\) uniporter (31–33) and the rapid 
Ca\(^{2+}\) uptake mechanism detected in liver and heart 
mitochondria (34). The currently 
known pathways responsible for 
mitochondrial Ca\(^{2+}\) efflux under 
physiological conditions include 
the mitochondrial Na\(^{+}/Ca^{2+}\) exchanger, which is predominantly 
expressed in excitable tissues, such 
as neuron and muscle, and the 
H\(^+/Ca^{2+}\) exchanger, which is 
mainly detected in nonexcitable tis-
tsues, such as liver and kidney (31, 
35). In addition to the specific Ca\(^{2+}\) transport pathways described 
above, opening of the mitochondrial 
permeability transition pore during 
apoptosis leads to efflux of sub-
stances with molecular mass of 
<1.5 kDa, including Mg\(^{2+}\), Ca\(^{2+}\), 
cytochrome c, adenine nucleotides, 
and mitochondrial matrix proteins, 
with the resulting collapse of \(\Delta \psi\) 
and mitochondria swelling (36). 
Currently, the molecular identities 
of the mitochondrial Ca\(^{2+}\) trans-
port pathways have not been deter-
mined. Using two-dimensional gel 
electrophoresis and proteomic 
analysis, we show here that the 
molecular chaperone hsp90\(\beta\) is 
reduced parallel to the loss of 
PIMCE during RA-induced NT2 
cell differentiation (Figs. 2 and 3 and 
Tables 1 and 2). The inhibition of 
PIMCE by 17-DMAG and hsp90\(\beta\) RNAi 
further confirms that 
hsp90\(\beta\) is required for PIMCE 
(Figs. 4–6). The inhibitory effect of 
17-DMAG, like that of hsp90\(\beta\) RNAi, may result from down-
regulation of hsp90\(\beta\) protein levels, 
since prolonged treatment with 
other geldanamycin derivatives has 
been shown to cause degradation of 
hsp90\(\beta\) proteins (37). The results 
of immunofluorescence experi-
ments in the current work demon-
strated that treatment with 
17-DMAG and hsp90\(\beta\) RNAi 
reduced protein levels of hsp90\(\beta\) in 
NT2 cells (Fig. 7). Previous studies 
have revealed differential roles for 
the N or C terminus of hsp90\(\beta\) in 
interaction with client proteins (22). 
We thus compared the acute effect
of 17-DMAG (which binds to the N terminus of hsp90β) and novobiocin (which binds to the C terminus) on PIMCE and found that only novobiocin effectively inhibited LA-induced PIMCE (Fig. 8). The distinctive effects of inhibitors binding to the N or C terminus of hsp90β on PIMCE in isolated mitochondria suggest that the C terminus of hsp90β plays a critical role in PIMCE. How hsp90β functions as a molecular constituent of the mitochondrial Ca^{2+} transporters activated by PUFA is currently unknown and is the subject of ongoing study in our laboratory.

The lack of PIMCE in brain mitochondria was unexpected, although the rate and amplitude of PIMCE in mitochondria were found to differ among other tissues tested (Fig. 1). Comparisons of component proteins and oxidative phosphorylation capacities indicate substantial diversities among the mitochondria of different rat tissues (muscle, heart, liver, kidney, and brain) (38, 39). The lack of PIMCE may be a unique feature of brain mitochondria. If hsp90β is a component of the pathway responsible for PUFA-induced mitochondrial Ca^{2+} transport, we would expect brain mitochondria to lack or have low levels of hsp90β proteins. Although the expression level of hsp90β in brain mitochondria was not directly investigated in the present work, diminished hsp90β levels detected upon RA-induced differentiation of NT2 neurons (Fig. 3) provided suggestive evidence that mitochondria of neural systems might contain less hsp90β compared with other tissues, such as liver and spleen.

We found that the inhibitory effect of 17-DMAG added to NT2 cells for 48 h was concentration-dependent but biphasic (i.e., at concentrations of 17-DMAG ≥10−8 M, the inhibitory effect on the LA-induced [Ca^{2+}]_{i}, signal was partially lost) (Fig. 4). The reduced inhibitory effect of 17-DMAG at high concentrations was associated with decreased NT2 cell numbers, which may occur through induction of cell death or arrested cell growth. The larger LA-induced [Ca^{2+}]_{i} signal in cells treated with high concentrations (≥10−8 M) of 17-DMAG may reflect elevated [Ca^{2+}]_{m} during apoptosis. We have previously shown that during apoptosis, G protein-coupled receptor-mediated [Ca^{2+}]_{i} signaling is inhibited (40). Thus, the reduction of carbachol-induced [Ca^{2+}]_{i} signal observed at high concentrations (≥10−8 M) of 17-DMAG may indicate that NT2 cells undergo apoptosis in the presence of high concentrations of 17-DMAG.

It has been shown that the hsp90 family is composed of multiple isoforms and is involved in diverse cellular processes, such as signal transduction, protein folding, and mitochondrial protein import (22, 41). Our data demonstrate that hsp90β plays an essential role in PIMCE.

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FIGURE 8. Acute effects of 17-DMAG and novobiocin on LA-induced Ca^{2+} efflux in mitochondria. LA (3.0 × 10^{-5} M)-induced Ca^{2+} efflux in mitochondria isolated from NT2 cells was measured in the absence (A) and presence of 17-DMAG (3.0 × 10^{-6} M) (B) or novobiocin (2.0 × 10^{-3} M) (C). Traces are from a typical experiment.
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