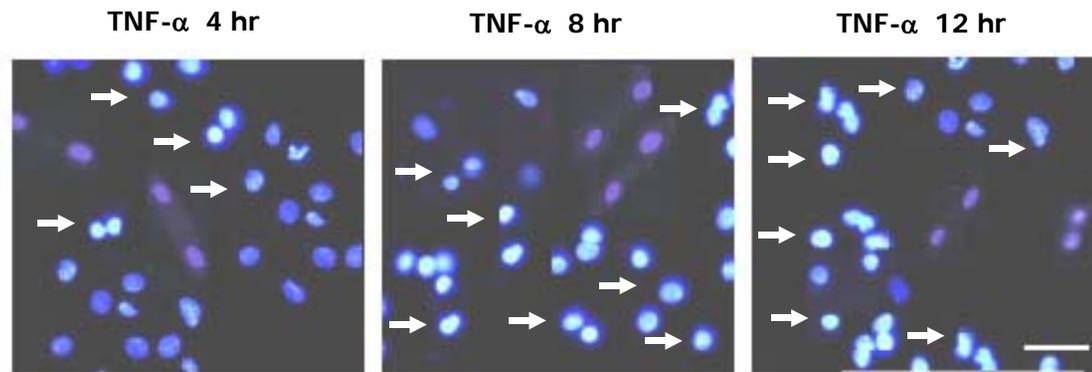
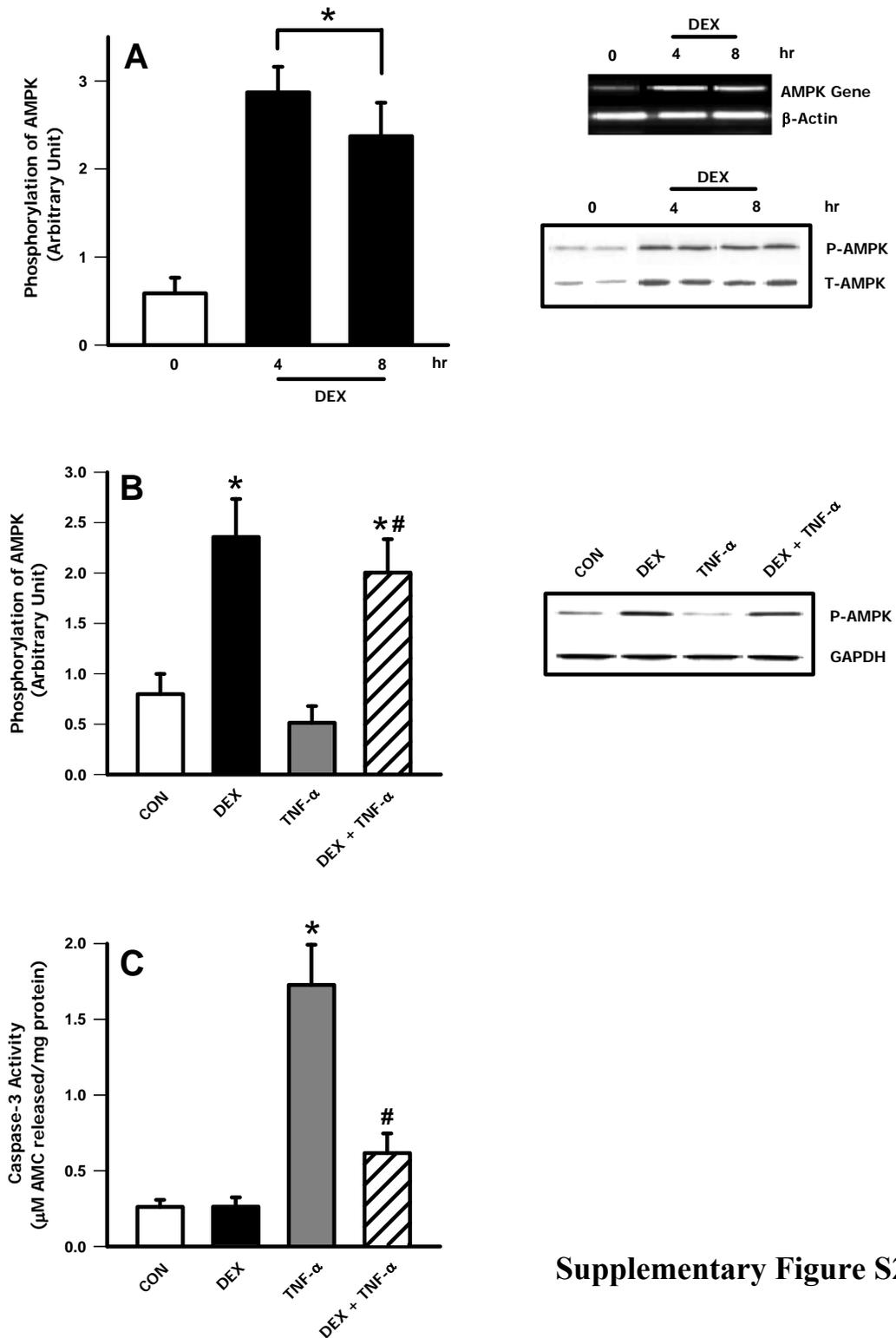


Supplementary Figure S1



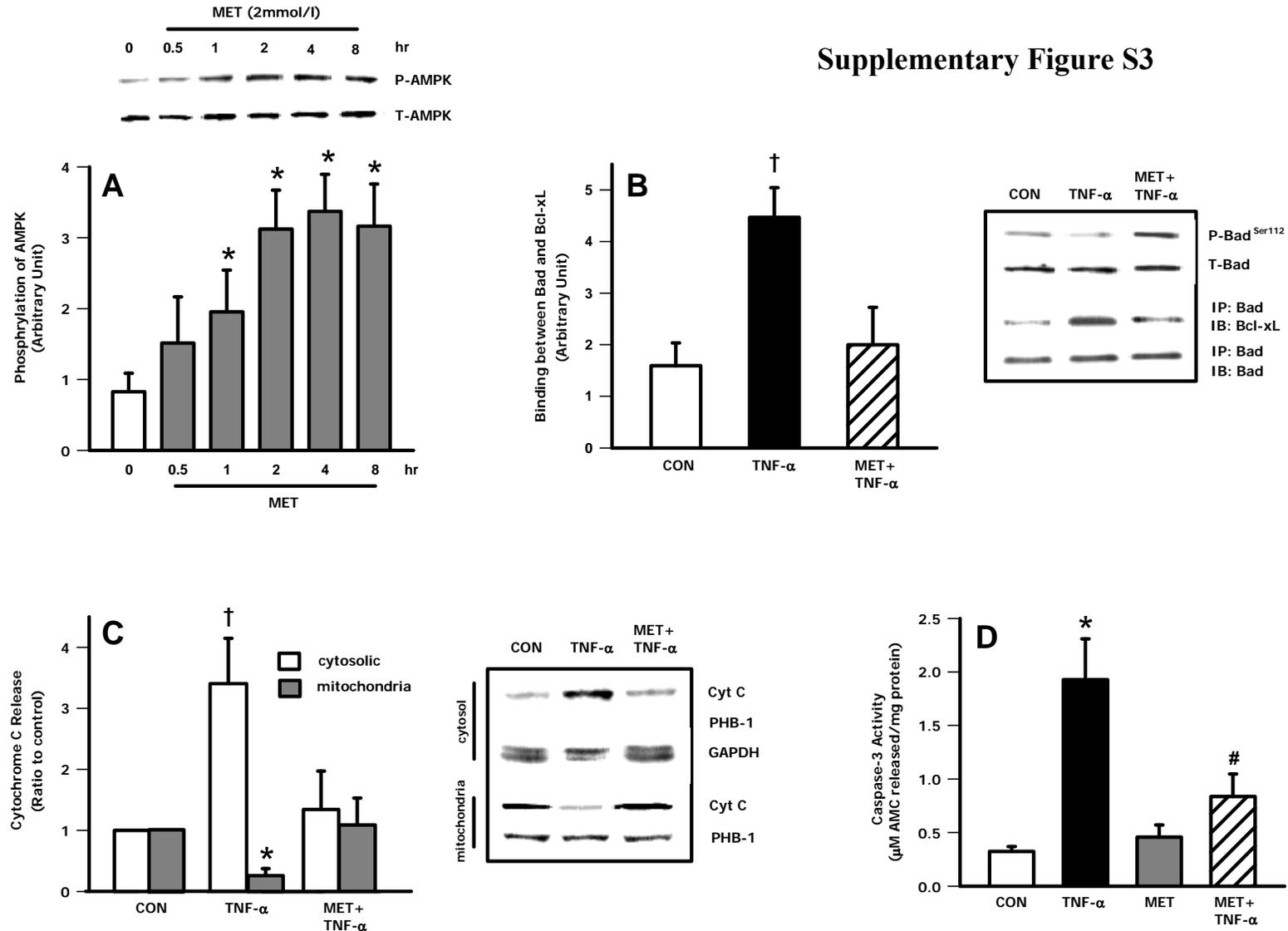
TNF- α induced cardiomyocyte apoptosis. Ventricular cardiomyocytes were exposed to TNF- α (10 ng/ml) for 4, 8 and 12 hr and morphological evidence of apoptosis using the fluorescent DNA-binding dye Hoechst 33342 were determined. Cells were scored as apoptotic (arrow) if they exhibited unequivocal nuclear chromatin condensation and/or fragmentation. Bar = 25 μ m



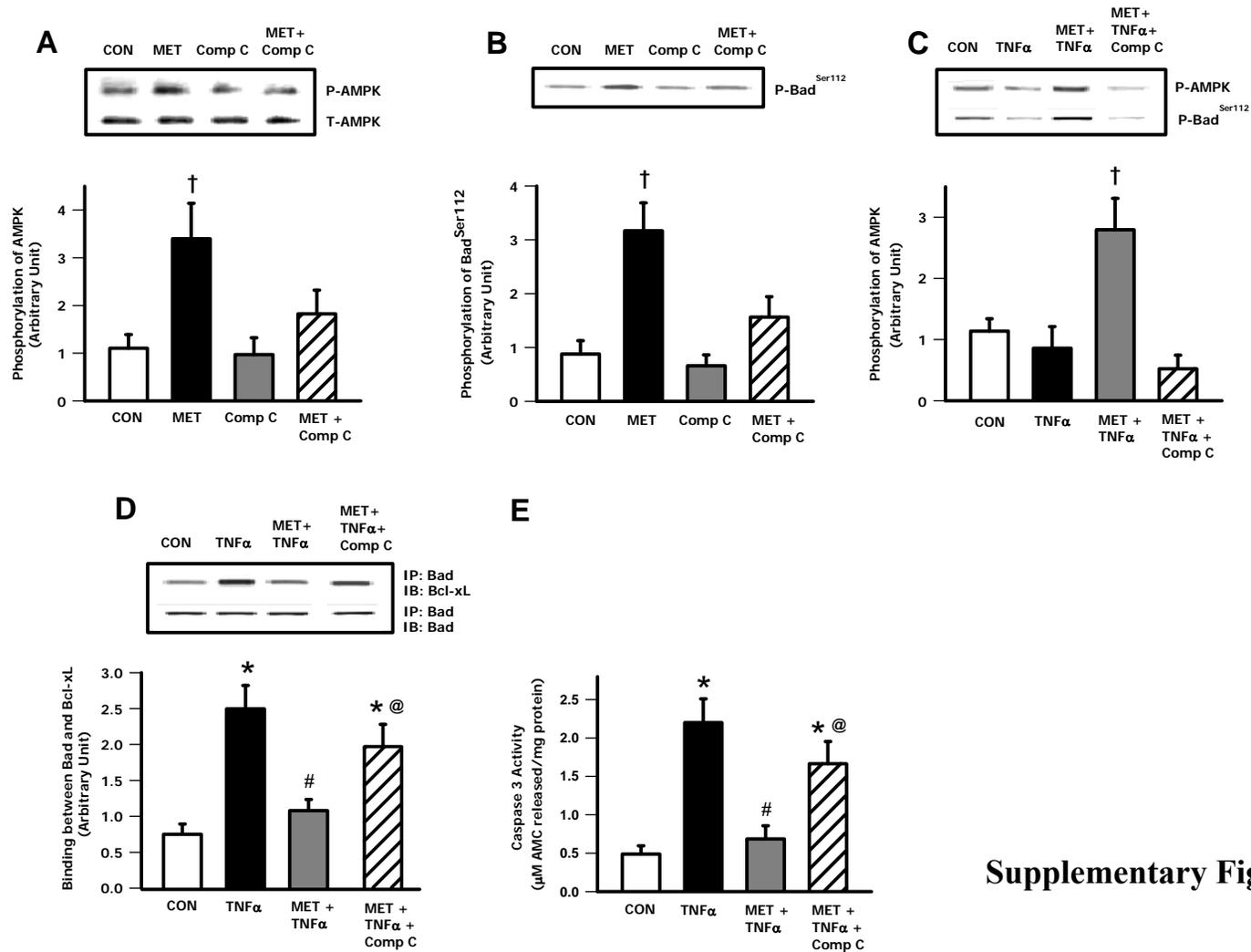
Supplementary Figure S2

***In vivo* effects of DEX administration in on TNF- α induced cell death *in vitro*.** DEX (1 mg/kg) was administered i.p and the animals killed 4 hr later. Hearts removed were used either for determination of whole heart AMPK or preparation of cardiomyocytes. Cells were plated for 8 hr in the presence or absence of TNF- α . Results are means \pm SE of 4 myocyte preparations in each group. *Significantly different from control (CON), #Significantly different from TNF- α . $P < 0.05$.

Supplementary Figure S3



Metformin induced regulation of Bad phosphorylation, its association with BCL-xL and its influence on caspase-3 activity. Control cardiomyocytes were incubated with TNF- α (8 hr) in the presence or absence of MET (2 mmol/l, 8 hr). Results are means \pm SE of 4 myocyte preparations in each group. *Significantly different from control (CON), [†]Significantly different from all other groups, [#]Significantly different from TNF- α , $P < 0.05$. MET, metformin; P, phosphorylated; T, total.



Supplementary Figure S4

Consequences of AMPK inhibition on the protective effects of metformin. Cardiomyocytes were pre-incubated with Compound C (40 μM) for 45 min, prior to MET (2 mmol/l) treatment for 8 hr. Results are means ± SE of 4 myocyte preparations in each group. *Significantly different from control (CON), #Significantly different from TNF-α, @Significantly different from MET+TNF-α, †Significantly different from all other groups, $P < 0.05$. MET, metformin; P, phosphorylated; T, total; IP, immunoprecipitated; IB, immunoblot.

SUPPLEMENTAL METHODS

Isolation of cardiomyocytes

Briefly, myocytes were made calcium-tolerant by successive exposure to increasing concentrations of calcium. Our method of isolation yields a highly enriched population of calcium-tolerant myocardial cells that are rod-shaped (over 80%) in the presence of 1 mmol/l Ca^{2+} , with clear cross striations. Intolerant cells are intact but hypercontract into vesiculated spheres. Yield of myocytes was determined microscopically using an improved Neubauer haemocytometer. Myocyte viability was assessed as the percentage of elongated cells with clear cross striations that excluded 0.2% trypan blue. Cardiomyocytes were plated on laminin-coated six-well culture plates to a density of 200,000 cells/well. Following time for adhesion (3 hr), cells were counted again (approximately 160,000 cells adhere/plate), and incubations initiated. Cells were maintained using Media-199 at 37°C under an atmosphere of 95% O_2 /5% CO_2 .

Administration of DEX to control rats

To examine whether DEX administration *in vivo* would maintain its protective effect against TNF- α induced cell death *in vitro*, DEX (1 mg/kg) or an equivalent volume of ethanol was administered by intraperitoneal injection and the animals killed 4 hr later (plasma half-life of DEX is ~279 min). The hearts removed were used either for determination of whole heart AMPK or preparation of cardiomyocytes. For the latter procedure, myocytes from control and DEX treated hearts were plated for 8 hr in the presence or absence of TNF- α .

Estimation of cardiomyocyte intracellular free Ca²⁺

Briefly, cardiomyocytes were plated on glass cover slips with etched grids. Plated cardiomyocytes were then transferred to an imaging chamber mounted on a temperature-controlled stage and held at 37°C on a Zeiss Axiovert 200 M inverted microscope equipped with a FLUAR 20× objective (Carl Zeiss, Thornwood, NY). For Ca²⁺ measurements, cells were loaded for 30 min with 5 μM of the Ca²⁺ sensitive dye Fura-2 (Molecular Probes/Invitrogen) in Ringer's solution containing (in mM): NaCl 144, KCl 5.5, MgCl₂ 1, CaCl₂ 2, Hepes 20 (adjusted to pH 7.35 by NaOH) and washed twice to remove any extracellular dye. Fura-2 was excited at 340 nm and 380 nm and the emitted fluorescence was monitored through a D510/80m filter. The Ca²⁺ levels at rest as well as maximal increases evoked by DEX were determined and expressed as the ratio of the fluorescence emission intensities (F₃₄₀/F₃₈₀).

Measurement of apoptosis

To examine TNF-α induced apoptosis and the influence of DEX and MET on this process, cells were examined for a) morphological evidence of apoptosis using the fluorescent DNA-binding dye, Hoechst 33342 (Sigma) or b) changes in caspase-3 (enzyme that plays a key role in apoptosis) activity using an assay kit or Western blot. Cells were stained with 5 μg/ml Hoechst 33342, and viewed on a Zeiss IM fluorescence microscope (×400) (Carl Zeiss Canada, Toronto, ON, Canada). Cells were scored as apoptotic if they exhibited unequivocal nuclear chromatin condensation and/or fragmentation. To quantify apoptosis, 500 nuclei from three different myocyte preparations were randomly picked and examined, and the results presented as apoptotic cells per 1,000 cells. Caspase-3 activity was determined using a fluorescent caspase-3

assay kit. Briefly, myocytes were lysed and protein extracted by centrifugation at 5,000 g for 5 min. 50 µl of protein were added to an equal volume of reaction buffer that contained 50 µmol/l of the respective substrate, and incubated at room temperature for 30 min. The enzyme-catalyzed release of aminomethylcoumarin was quantified in a fluorimeter (Perspective Biosystems, Framingham, MA, USA) at 380/450-nm wavelengths. Protein was determined using a Bradford assay (Bio-Rad, Hercules, CA, USA).

Western Blot

Briefly, plated myocytes (0.4×10^6) were homogenized in ice-cold lysis buffer. After centrifugation at 5,000 g for 20 min, the protein content of the supernatant was quantified using a Bradford protein assay. Samples were diluted, boiled with sample loading dye, and 50 µg used in SDS-polyacrylamide gel electrophoresis. After transfer, membranes were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween-20. Membranes were incubated with rabbit total AMPK- α , phospho-AMPK (Thr-172), phospho-ACC, Bcl-xL, Prohibitin-1 (PHB1), GAPDH, cleaved caspase-3 (Asp175), caspase-3, total Bad, phospho-Bad (Ser112), phospho-CAMKI (Thr177), total CaMKI and cytochrome C antibodies and subsequently with secondary goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antibodies, and visualized using the ECL detection kit (Amersham).

Cardiomyocyte AMPK gene expression

Briefly, total RNA from control and treated cardiomyocytes was extracted using TRIzol (Invitrogen), and reverse transcription was carried out using an oligo(dT) primer and SuperScript II RT (Invitrogen). cDNA was amplified using AMPK [5'-

GCTGTGGATCGCCAAATTAT-3' (*left*) and 5'-GCATCAGCAGAGTGGCAATA-3' (*right*)]-specific primers. The β -actin gene was amplified as an internal control using 5'-TGGTGGGTATGGGTCAGAAGG-3' (*left*) and 5'-ATCCTGTCAGCGATGCCTGGG-3' (*right*). The amplification parameters were set at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min for a total of 30 cycles. The PCR products were electrophoresed on a 1.7% agarose gel containing ethidium bromide. Expression levels were represented as the ratio of signal intensity for AMPK mRNA relative to β -actin mRNA.