IGF-I activates PKB and prevents anoxic apoptosis in Achilles tendon cells

Alexander Scott, **Karim M. Khan**, and **Vincent Duronio**[*]
Department of Medicine, University of British Columbia and Vancouver Coastal Health Research Institute, Vancouver, BC, Canada, V6H 3Z6

Abstract

Anoxia and apoptosis are both implicated in chronic tendon pathology, however the influence of anoxia on the viability of tendon cells is not known. The objectives of the current study were to (i) investigate the effect of oxygen withdrawal on the viability of porcine Achilles tendon cells (ATCs), and (ii) examine the ability of IGF-I, a factor with known regenerative properties in tendon, to prevent ATC death. Cultured ATCs were enclosed in an anaerobic chamber. The mechanism of cell death was examined by flow cytometry of ATCs double labeled with Annexin-V and propidium iodide (PI). Caspase activity was determined by a fluorometric assay, and nuclear morphology was examined by Hoechst staining. The cell death induced by anoxia was time-dependent, and was characterized by phosphatidylserine exposure on the outer membrane, caspase activation and DNA fragmentation. Death was inhibited by the addition of IGF-I in a dose-dependent manner. The ability of IGF-I to activate the pro-survival PKB pathway in ATCs was inhibited by LY294002, indicating the importance of PI3K in the response of ATCs to IGF-I. These data suggest that cell death induced by lack of oxygen is predominantly apoptotic and can be prevented by pro-survival IGF-I signaling. This mechanism may contribute to the beneficial effect of IGF-I on tendon.

Keywords

Achilles tendon; anoxia; IGF-I; apoptosis

Introduction

Achilles tendon injury is an important clinical problem in both athletes and non-athletes (24, 27). The histopathology of ruptured tendons shows evidence of pre-existing pathology that predisposes to rupture (35). Of various patterns of pathology including hypoxic, mucoid, fatty, and calcific tendinopathies, hypoxic tendinopathy was the most common in a series of 891 tendon ruptures (23). Although the histology of hypoxic tendinopathy is well described (including regions of hypocellularity, pyknotic nuclei, necrosis, mitochondrial abnormalities, and collagen thinning and disarray) (23),the underlying cellular processes are poorly understood.

[*]To Whom Correspondence and Reprint Requests Should Be Addressed: Dr. Vincent Duronio, Jack Bell Research Centre, 2660 Oak St., Vancouver, B.C., V6H 3Z6, CANADA, Telephone: 604 875-4707, Fax: 604 875 4497, vduronio@interchange.ubc.ca.

Apoptosis, or programmed cell death, is associated with chronic tendon pathology (46, 47). In patients with supraspinatus rupture and with tennis elbow, apoptosis has been reported based on TUNEL staining, DNA laddering and transmission electron microscopy (20, 47). Other features of apoptosis that distinguish it from classic cell death (necrosis) include the activation of pro-death proteases – caspases – and the exposure of phosphatidylserine on the outer plasma membrane that serves as a signal for phagocytosis by macrophages (4, 18, 25). Although apoptosis plays important homeostatic and physiologic roles (for example during regression of embryonic structures) it also plays negative roles in degenerative conditions of bone, cartilage, muscle and nerve. In general, the cell's decision to survive or to commit to apoptosis appears to result from a balance of pro-survival and death-inducing signals (8).

The events leading to tendon cell death or survival are beginning to be explored (46, 47) but the influences of hypoxia and growth factors on tendon cell viability have not been examined. In other cell types, hypoxia and anoxia induce necrosis, or apoptosis via transcription pathways involving p53 and p38 (13, 40). Opposing such damage following injury is IGF-I a prosurvival factor released by platelets, macrophages, and fibroblasts (19, 37–39). By binding to the IGF-I receptor, IGF-I enacts a number of pro-survival signaling events, including activation of protein kinase B (PKB) which prevents cell death by phosphorylating various cytoplasmic and nuclear targets (44). The effects of IGF-I on tendon cells are of special interest given its promise as a factor that can accelerate biochemical and biomechanical recovery after tendon injury (1–3, 9, 14, 26, 29).

Given that reduced oxygen may play a role in acute or chronic Achilles tendinopathies (12, 21, 33) and that IGF-I has documented anabolic and regenerative effects in tendon (1–3, 9, 14, 26) we evaluated the influence of anoxia and IGF-I on death and survival signal pathways in Achilles tendon cells. The specific objectives were to determine (i) the extent and type of cell death induced by anoxia, and (ii) the ability of IGF-I to inhibit anoxia-induced cell death and to activate the PI3K/PKB pathway.

Materials and Methods

Reagents

Type I collagenase (clostridiopeptidase A), IGF-I, and avidin-flourescein isothiocyanate (FITC) were purchased from Sigma (St Louis, MO). Heat-inactivated fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). Annexin-V conjugated to biotin was obtained from Pharmingen (San Diego, CA). Anaerobic chambers, pouches and indicator strips were supplied by BD (Gaspak, Franklin Lakes, NJ). A primary rabbit polyclonal antibody detecting the phosphorylated form of PKB was from Stressgen (Victoria, Canada). P85 antibody was obtained from Cell Signaling Technologies (Beverly, MA).

Achilles tendon cell culture

Porcine Achilles tendon tissue, harvested aseptically from animals being euthanized for university teaching purposes, was placed immediately into sterile PBS. Adherent muscle and peritendinous tissue was removed. The sample was then washed repeatedly in sterile PBS, cut into 0.5 mm pieces with a sterile blade and forceps and digested for 24–48 hours in

DMEM containing 0.5mg/ml collagenase, 10% FBS, (v/v) 20 mM Hepes, pH 7.4, 2% (v/v) penicillin-streptomycin, and 0.1% (v/v) gentamycin on an agitator at 37°C. The tendon fibroblasts released by this procedure were plated, along with any undigested collagenous tissue, on plasma-treated polystyrene tissue culture dishes. Tendon cell cultures were maintained in DMEM with 10% (v/v) FBS in a 5% CO_2 incubator at 37°C. Cells were used in experiments up to the fifth passage.

To examine the effects of anoxia, tendon cells were seeded at 500,000 cells per 60mm plate and allowed to adhere for 48–72 hours, at which point the FBS concentration was reduced to 1%; cells were then maintained as usual in the 5% CO_2 incubator (normoxia) or placed at 37°C in an anaerobic chamber (GasPak system) for 24–96 hours (anoxia). Anoxia was confirmed by using methylene-blue indicator strips which become colorless when oxygen is absent. All experiments were performed in triplicate.

Cell viability assay

After indicated timepoints of normoxia or anoxia, cells were harvested by trypsinization and scraping, washed in PBS, fixed for 1h in ice-cold 70% ethanol, then placed for 30 minutes in 50μg/ml propidium iodide (PI) in PBS with 0.1% glucose and 100μg/ml RNaseA. A histogram representing DNA content was generated from each sample using the FL3 channel on a flow cytometer (Coulter Epics XL-MCL); the percentage of apoptotic cells (with sub-diploid DNA) was quantified using the same gate for all samples.

Apoptosis assay

To analyze the mechanism of tendon cell death, normoxic and hypoxic (anoxic) cells were harvested and washed as above, then resuspended in 500 μl of 10mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4 to which 5μl of biotinylated Annexin V was added. After 15 minutes incubation in the dark, 5μl of Avidin-FITC (50μg/ml) and 5μl of PI (10mg/ml) were added. After 30 minutes, the samples were analyzed on a flow cytometer simultaneously on the FL1 and FL3 channels after appropriate compensation with single-stained and unstained tendon cells. Each channel was gated, yielding a scatter plot with four quadrants representing cells that were viable, in early or late apoptosis, or necrosis (46).

Caspase activation assay

Caspase activation was examined in live and dying cells using a flourometric assay kit according to the manufacturer's instructions (Immunochemistry). Briefly, a fluorescent probe (FAM-VAD-FMK FLICA) was added to anoxic or normoxic tendon cells harvested as above. The probe diffuses freely across the plasma membrane and reacts broadly with active enzymes of the caspase family by binding covalently to a reactive cysteine residue on the active caspase (43). After several washes in PBS, the cells were analyzed by flow cytometry and on a fluorescent microscope (Zeiss Axioplan) after a 10 minute incubation with Hoechst 33342, a nuclear dye which is more readily taken up by apoptotic cells (10).

Western blot analysis for PKB activation

Tendon cells were harvested and 500,000 cells were allowed to adhere in 60mm plates overnight. To render cells quiescent prior to IGF-I stimulation, serum was withdrawn for 12

hours, then IGF-I was added to the medium for varying concentrations and durations. Cells were released from the plate by scraping, centrifuged and lysed in ice-cold solubilization buffer containing 50mM Tris-HCL, 1% Triton X-100, 10% glycerol, 100mM NaCl, 25mM EDTA, 10mM NaF, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates were sonicated on ice for 10 seconds, centrifuged at 14,000 rpm for 10 min, boiled in SDS and beta-mercaptoethanol, then loaded onto a 9% SDS-PAGE separating gel. Gels were calibrated using prestained SDS-PAGE low molecular weight standards. Proteins were transferred electrophoretically to nitrocellulose membranes, blocked for 1 h in Tris-buffered saline (TBS) containing 3% skim milk, then incubated overnight with the primary anti-PKB antibody at room temperature. After three washes with TBS and 0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 1 h. Bands were visualized by using enhanced chemiluminescence. Equal loading was confirmed by reprobing the membranes with antibodies recognizing the p85 subunit of PI 3-kinase.

Statistical analysis

All values in the text and figures are expressed as mean ± S.D. of 3 observations. Statistical comparison between experimental group and control was performed using a repeat measures ANOVA. The confidence limit was predetermined at an α level of 0.05.

Results

Oxygen withdrawal induces apoptosis of Achilles tendon cells

Lack of oxygen resulted in a progressive increase in the rate of cell death. However, the rate of cell death did not reach substantial levels until after 48 hours (Figure 1). Morphologically, the majority of dying cells demonstrated features of apoptosis (shrunken cytoplasm, membrane blebbing) rather than necrosis (swollen cytoplasm, membrane disruption). In cells kept under anoxic conditions but in the presence of added IGF-1, there was no evidence of an increase in cell death over the time period examined (up to 96 hours). Flow cytometry of cells double labeled with Annexin-V and PI confirmed that the predominant type of cell death induced by anoxia was apoptosis (Figure 2A&B). Among the dead cells (PI positive), the majority were Annexin positive (apoptotic), with few being Annexin negative (necrotic).

Caspase activation in anoxic tendon cells

As some authors refer to a "caspase-independent" form of programmed cell death ("aponecrosis")(4), we undertook experiments to confirm that cell death in these experiments was accompanied by caspase activation. Exposure of tendon cells to anoxia increased caspase activity 3- to 4-fold compared to cells treated identically but kept at normal oxygen tension, as evidenced by flow cytometry (Figure 2C&D). The finding of caspase activation was further validated by labeling anoxic tendon cells (72 hours) simultaneously with the same fluorometric caspase probe and with Hoechst 33342. Tendon cells that demonstrated green fluorescence in the cytoplasm, indicating active caspases, frequently showed increased uptake of Hoechst and fragmented nuclear morphology (Figure 2F).

IGF-I prevents anoxic cell death

ATCs in which IGF-I was added at the same time that oxygen was withdrawn demonstrated levels of cell death equivalent to control values up to 96 hours. The dose-response effect of IGF-I on cell survival was tested using concentrations of IGF-1 up to 500ng/ml (Figure 3). A maximal survival effect was observed at a concentration of 250 ng/ml.

IGF-I activates PKB through a PI3K dependent manner

To investigate one of the likely mechanisms of anti-apoptotic signaling by IGF-I in tendon cells, a phospho-specific antibody was employed that recognizes PKB when phosphorylated at the Ser473 activation site. Immunoblotting revealed a rapid increase in phosphorylation of PKB, which was elevated by $2'$, peaked at 5 minutes and had nearly disappeared by 20 minutes (Figure 4). The phosphorylated form of PKB was not detectable in cells that were pre-incubated with LY294002, a known PI3K inhibitor. LY294002 (25μm) also caused substantial cell death when added to normoxic, quiescent cell cultures indicating that ongoing survival of ATCs is dependent on the PI3K – PKB pathway (data not shown).

Discussion

In response to a lack of oxygen, Achilles tendon cells undergo either apoptosis or necrosis, with apoptosis representing the predominant mode of death. Apoptosis has not previously been well characterized in tendon cells. This study showed that it occurs in a manner that is characteristic of apoptosis in other cell types that includes exposure of phosphatidylserine on the outer plasma membrane, caspase activation, and nuclear fragmentation (4).

We examined the effects of complete anoxia on tendon cells rather than gradations of PO_2, in order to induce a large effect that would allow us to determine whether oxygen withdrawal would induce apoptosis. In young rats, the mean PO_2 of tendons was 78.6 mmHg (34), but the PO_2 of aged, injured, or hypovascular regions of Achilles and other tendons are not known. Because oxygen supply is known to be a key determinant of a successful healing response (42), future studies to determine actual PO_2 in regions of tendinosis before or after rupture are required. Further experiments should also examine the effects of different hypoxic conditions with gradations of PO_2.

Although tendon cells express a functional aerobic metabolism (11), they are relatively insensitive to oxygen withdrawal compared to other cell types. Tendon explants maintained in 0% O_2 in 10% FBS for 48 hours demonstrated no decline in cell proliferation or proteoglycan production, but collagen production was inhibited; this was in contrast to synovial explants, in which proliferation was significantly reduced, along with proteoglycan and collagen production (31). Likewise, cultured human embryonic Achilles tendon cells whose oxygen was reduced from 20% to 2.7% did not experience a reduction in proliferation, whereas fibroblasts derived from other tissues did (45). Furthermore, the magnitude of cell death we report here for tendon cells is several times lower than that reported in similar experiments with various cell types (7, 17, 30, 40). The relative insensitivity of tendon fibroblasts to hypoxia raises the question of why the relatively low percentage of cells undergoing apoptosis at 72 hours (15–20%) were less resistant than the

cells which survived for this duration. From other cell types, hypoxia has been found to irreversibly damage some cells, while surviving cells from the same initial population can exhibit adaptive changes which increase their resistance to hypoxia and other stressful agents (16). Such investigations have yet to be carried out in tendon cells, and may be relevant in understanding their resistance to cell death as shown in the current study.

Chronic Achilles tendon pathology is associated with an angiogenic response (6). Animal studies indicate that angiogenesis represents a late feature of overuse pathology, with neovessels taking several weeks or months to form and penetrate the area of pathology (28). The increased lactate level in Achilles tendinosis (5) suggests that angiogenesis may lag behind oxygen requirements. It is tempting to speculate that compromised blood supply leading to a gradation of hypoxia (with the most severe levels at the center of a tendon lesion) could account for the histological appearance of a central zone of cell death bordered by a peripheral zone of proliferation (45). This pattern is also seen in tumours, which display a central zone of hypoxia, despite an active angiogenic response. Hypoxia could lead not only to cell death, but could also influence transcription in tendon cells resulting in matrix abnormalities such as fibrocartilaginous change, calcification and bony metaplasia (15). These speculations must be tested empirically as the actual conditions within injured tendons are likely to be very complex, with numerous interacting conditions and factors (including mechanical loading, pH, and soluble or matrix-bound substances) determining the outcome.

Chronic hypoxia, one of many stresses that can trigger apoptosis, appears to operate through a pathway that is distinct from most stress-inducing agents (32). Under acute anoxic conditions, the level of hypoxia-inducible factor (HIF-1α) - the "cellular sensor" of oxygen levels - rises, and activates transcription of genes involved in the response to hypoxia, including those encoding TGF-beta and VEGF. If oxygen is chronically low, HIF-1α binds and stabilizes p53, a tumor-suppressor transcription factor capable of inducing cell-cycle arrest or apoptosis. P53 is known to be elevated in experimentally ruptured tendon (22). Hypoxia may also induce apoptosis through other pathways; for example, one report suggested that hypoxia-induced apoptosis requires the development of acidosis (36), but in our experiments apoptosis occurred with no change in the pH of the medium. In vivo, tendons undergo mechanical strain, and static tension held over several minutes reduces tendon oxygen levels (41). It is unlikely that tension-induced hypoxia would induce apoptosis in tendons, as periods of complete hypoxia (anoxia) >24 hours were required to induce substantial amounts of apoptosis in the current study.

In summary, our studies demonstrate that IGF-I activated the PKB pathway in tendon cells in a PI3-kinase dependent manner, and that IGF-I prevented anoxic cell death of ATCs. These findings extend studies conducted using other cell types, in which IGF-I exerts a protective effect from various cellular stresses. Whether other growth factors exert similar pro-survival effects in tendon cells is not yet known. We conclude that future studies should investigate the relative importance of downstream IGF-I signaling, and develop improved methods of achieving prolonged IGF-I delivery to areas of tendon pathology.

Acknowledgments

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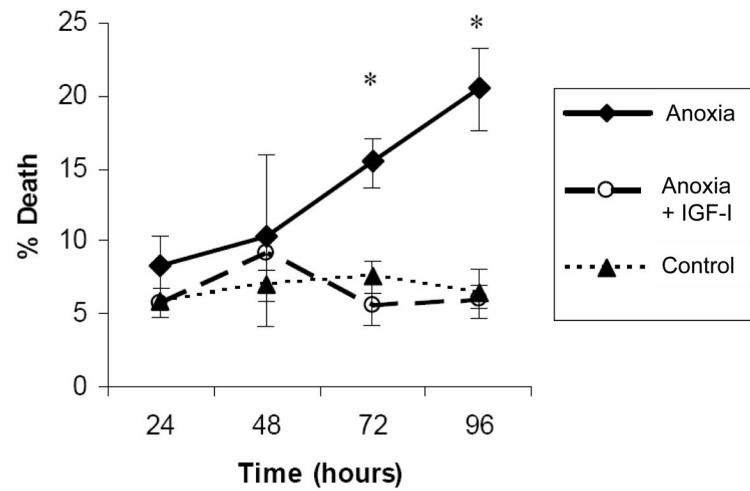
Figure 1.
Pro-survival effect of IGF-I under anoxic conditions. Cells were maintained under normoxic or anoxic conditions for the indicated time periods and cell death was indicated by the number of cells having sub-diploid DNA content. Incubation with IGF-I restored the level of tendon cell death to control levels at all time periods examined. * $p < 0.01$.
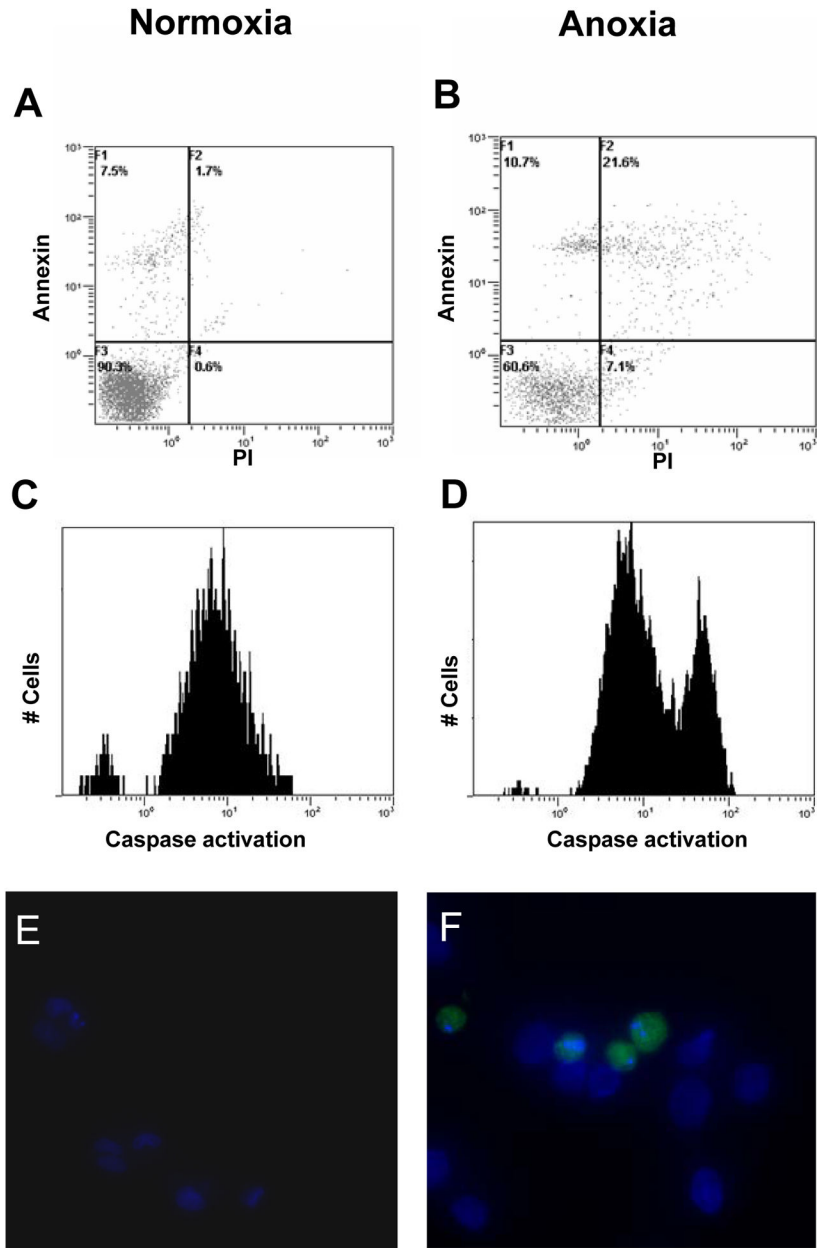
Figure 2.
Anoxia-induced tendon cell death is predominantly apoptotic. Cells were kept at normoxia (A, C, E) or anoxia (B, D, F) for 72 hours. A & B: Cells were trypsinized and double-labeled with PI and Annexin V. The bottom left quadrant represents viable cells that are negative for both probes. The top two quadrants represent early (left) and late (right) apoptotic cells, whereas cells at the bottom right are necrotic (PI+, Annexin−). C & D: Tenocytes were incubated with a fluorescent probe recognizing active caspases. In each panel, the largest peak represents background fluorescence from unstained cells. The right-most peak in the anoxic tencoytes (D) is due to positive labeling of tendon cells containing active caspases. E & F: Tendon cells with active caspases (green) were common in the 72 hour anoxic cultures.

These same cells frequently demonstrated increased uptake of Hoechst 33342 and fragmented nuclei, indicating that they were undergoing apoptosis.
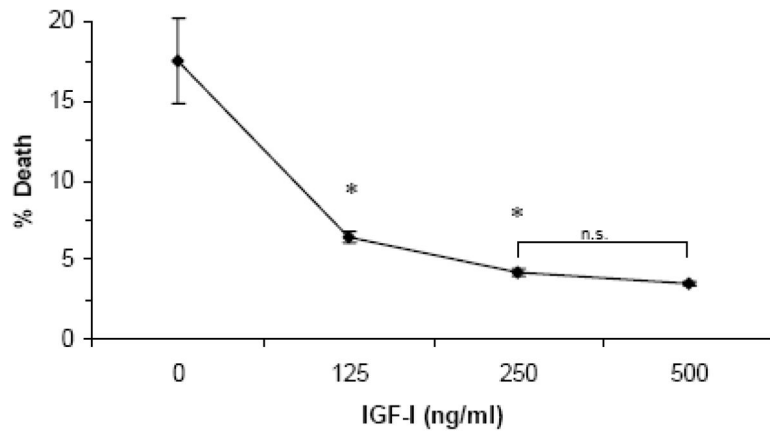
Figure 3.
Dose-response of IGF-I protection from apoptosis during anoxia (72 hours). * indicates significant difference from controls and from each other, $p < 0.001$. N.S; not significantly different from one another.
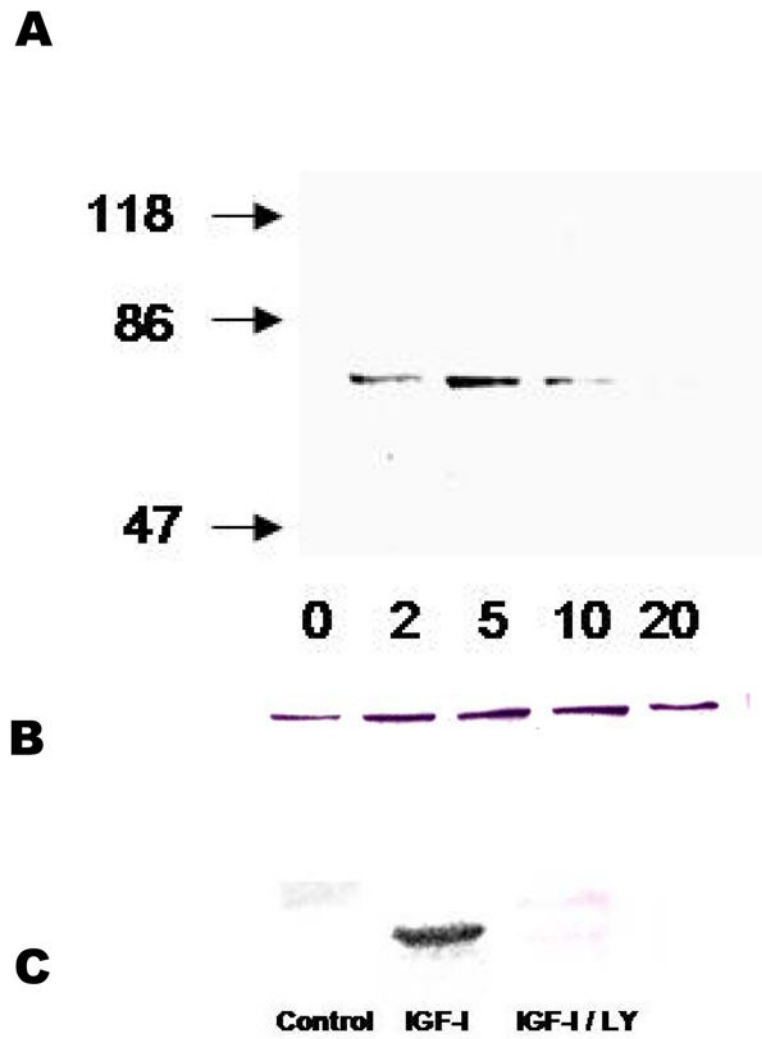
Figure 4.
Activation of PKB by IGF-I. A: Phosphorylation at Ser473 showed a rapid onset and decay. B: The same membrane probed with P85 demonstrating equal loading. C: Inhibitory effect of LY (25μm) on PKB activation.