Expression of Adrenomedullin and Proadrenomedullin N-Terminal 20 Peptide in PC12 Cells after Exposure to Nerve Growth Factor

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Abbreviations used: AM, adrenomedullin; cAMP, cyclic AMP; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ir, immunoreactive; NGF, nerve growth factor; PAMP, proadrenomedullin N-terminal 20 peptide; SSC, saline-sodium citrate; TFA, trifluoroacetic acid.

Abstract

Adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP) are multi-functional peptides derived from the same precursor, proadrenomedullin. We have studied the regulatory mechanism of expression of these peptides during neuronal differentiation of rat pheochromocytoma PC12 cells by nerve growth factor (NGF). The cellular levels of the peptides increased slightly, and then progressively decreased below the control by NGF. Immunoreactive (ir)- AM in the medium was transiently increased by NGF. Cytochemical staining showed that ir-AM and ir-PAMP were abundantly present in cytoplasm in the undifferentiated cells, and were decreased during culture with NGF. There was no preferential localization of ir-AM or ir-PAMP in neurites in comparison with in cytoplasm in the differentiated cells. Northern blot analysis showed that mRNA encoding these peptides, as detected as a band of 1.6 kb, increased more than 3-fold at 1 hour after the addition of NGF and then progressively decreased to 1/5 of the control during 72 hours. Degradation rate of the mRNA was slowed by NGF even when mRNA level is decreased after 72 hours of NGF treatment. The transcription rate of their gene increased transiently and then decreased by the long-term treatment with NGF. These results demonstrate that expression of AM and PAMP is regulated by NGF along with time-dependent differentiation: AM gene transcription is transiently activated by NGF, whereas was suppressed during neuronal differentiation of the cells.

Key words: Adrenomedullin-PAMP-Nerve growth factor-Neuronal differentiation-Expression-PC12 cells.

Running title: NGF regulates adrenomedullin and PAMP expression

Introduction

Adrenomedullin (AM), originally discovered as a hypotensive peptide from human pheochromocytoma (Kitamura et al. 1993), now recognized as a multi-functional peptide. The precursor of AM, proadrenomedullin, produces also a biologically active peptide, proadrenomedullin N-terminal 20 peptide (PAMP) (Kitamura et al. 1994). PAMP caused hypotension as same as AM when intravenously injected in the rat (Kitamura et al. 1994), but the mechanism of action of PAMP is different from that of AM. AM dilates arteries by acting vascular smooth muscle directly via elevation of intracellular cAMP and by production of nitric oxide through elevation of intracellular calcium concentration in vascular endothelial cell (Shimekake et al. 1995). On the other hand, PAMP dilatates blood vessels by suppressing norepinephrine release from sympathetic nerve in rat mesenteric arteries (Shimosawa et al. 1995).

AM and PAMP are involved in the regulation of a variety of pathophysiological function not only of circulatory, respiratory and endocrine systems but also of the nervous system. AM administrated in intracerebroventricule activates oxytocin producing neurons, and increases plasma oxytocin level (Ueta et al. 2000). In addition, AM administrated in the central nervous system (CNS) causes hypertension, activation of sympathetic neuron innervating kidney (Takahashi et al. 1994; Saita et al. 1998), inhibition of drinking (Murphy and Samson 1995) and feeding (Taylor et al. 1996), and inhibition of salt feeding (Samson and Murphy 1997). Intracerebroventricular administration of PAMP also exerts hypertensive action via activation of the sympathetic nervous system like as AM (Samson et al. 1998). PAMP also produces hypoglycemia by acting gastrin-releasing peptide preferring receptor of bombesin receptor family (Ohinata et al. 2000). In addition, PAMP suppresses the nicotinic acetylcholine receptor response of the rat locus coeruleus neurons (Nabekura et al. 1998).

In the adrenal gland, AM and PAMP are synthesized and released from chromaffin cells, and they have different physiological roles. PAMP inhibits nicotinic cholinergic receptor function of the cell, and suppresses catecholamine synthesis and release as well as the induction of catecholamine synthesizing enzymes in an autocrine manner (Katoh et al. 1995). On the other hand, AM had no effect on catecholamine release from cultured bovine adrenal chromaffin cells or from perfused dog adrenal gland (Masada et al. 1999), thus the main function of AM may be the regulation of local blood flow or the remodeling of vasculature by regulating proliferation and apoptosis of

vascular smooth muscle cells and endothelial cells (Kobayashi et al. 2001a, 2003). However, it is not clear how the expression of these peptides is regulated.

AM and PAMP levels change in various physiological and pathological conditions. For example, AM and its mRNA levels in placenta and in maternal plasma increase during pregnancy (Di Iorio et al. 1997; Di Iorio et al. 1998; Kobayashi et al. 2000b). Severe exercise elevates the plasma AM levels with an increase in plasma norepinephrine and exaggerated sympathetic nerve activity (Tanaka et al. 1995). In the pathological conditions, the plasma AM and PAMP levels are increased in patient with a variety of diseases such as congestive heart failure (Jougasaki et al. 1995), myocardial infarction (Kobayashi et al. 1996), renal diseases (Ishimitsu et al. 1994), hypertension (Kita et al. 1998), diabetes mellitus (Hayashi et al. 1997) and septic shock (Hirata et al. 1996). Thus, the elucidation of regulatory mechanisms of these peptides is crucial not only for understanding the pathophysiological function of these peptides but also for their application for therapeutics and diagnosis of various pathological conditions clinically. However, information concerning to the regulatory mechanisms of AM and PAMP expression in the nervous system is very limited.

The rat pheochromocytoma PC12 cells engage a program of physiological changes resulting in a phenotype resembling that of sympathetic neurons by nerve growth factor (NGF) (Greene and Tischler 1976). Neuronal differentiation of PC12 cells modifies expression of several kinds of peptide, but their regulatory mechanism has not been fully understood. The aim of this study is to elucidate the regulatory mechanisms of expression of AM and PAMP during neuronal differentiation of PC12 cells.

Experimental procedures

Culture of PC12 cells

PC12 cells were cultured on poly-ornitine coated plastic dish at the density of 5 x 10^4 cells/cm^2 on 35 mm dishes or 10 cm dishes (Falcon, Tokyo, Japan) in RPMI1640 containing 30 μg/ml aminobenzyl penicillin, 50 μg/ml streptomycin, 5% fatal calf serum and 10% horse serum (JRH, KS) in the presence or absence of 2.5s NGF (50 ng/ml) (Alomone, Jerusalem, Israel), epidermal growth factor (EGF, Wako, Osaka, Japan), basic fibroblast growth factor (bFGF, Collaborative Biomedical, Bedford, USA), human insulin (Eli Lilly Japan, Kobe, Japan) or human platelet

derived growth factor-AA (PDGF, PeproTech EC, London, England).

Measurement of immunoreactive AM and PAMP

Cells were washed with ice-cold PBS, collected with 2 ml of 1 M acetic acid and heated at 100°C for 10 min. After centrifugation at 10,000 g for 15 min, the resulting supernatant was lyophilized and suspended in RIA buffer composed of 50 mM Na_2HPO_4, 25 mM EDTA, 80 mM NaCl, 0.05% NaN_3, 0.5% Triton X-100 and 0.5% BSA (Kobayashi et al. 1999). Immunoreactive (ir)-AM was measured in duplicate using antiserum against human AM[40-52]-NH_2 which recognizes the C-terminal amide structure of AM, whereas ir-PAMP was measured using antiserum against human PAMP[1-20] which recognizes entire PAMP molecule (Kobayashi et al. 1999). Both RIA systems had 100% cross reactivity with rat AM and PAMP but not with pre-proadrenomedullin that lacks C-terminal amide structure. From 1 to 120 fmol/tube of AM and PAMP was measurable by these RIA systems. The number of samples was 3 or 4 in each experiment, and the experiments were repeated at least 3 times. Protein content in the cells was measured according to the method of Lowry et al. (Lowry et al. 1951). The data are expressed as means $\pm$ SEM. One-way analysis of variance with post hoc mean comparison was used to test the statistical significance.

Northern blot

RNA was isolated from the cells by acid guanidine thiocyanate-phenol-chloroform extraction using TRIzol^{TM} (GIBCO BRL, Grand Island, NY, USA) and electrophoresed on 1% agarose gel containing 6.3% formaldehyde, 0.5 mM EDTA and 40 mM MOPS (pH7.4). RNA was transferred to nylon membrane (Hybond-N, Amersham) using 20 x saline-sodium citrate (SSC: 1 x SSC=0.15 M NaCl and 0.015 M sodium citrate) and fixed by UV radiation. The membrane was pre-hybridized at 42°C for 4 h in a hybridization buffer (6 x SSC, 10 x Denhardt's solution, 50% formamide, 0.5% sodium dodecyl sulfate, and 50 mg/ml salmon sperm DNA), and hybridized with Nae I fragment of rat AM cDNA (589 bp, D15069) labeled with [α-^{32}P]dCTP using Multi prime labeling kit (Amersham) at 42°C overnight. The membrane was washed in 2 x SSC containing 0.1% sodium dodecyl sulfate at 55°C for 1 h and then in 0.2 x SSC containing 0.1% sodium dodecyl sulfate at 65°C for 3 h, and subjected to autoradiography. After removing the probe, the membrane was re-hybridized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Clontech, Palo Alto, USA) probe. The intensity of the

band was estimated by Bio-Imaging analyzer (Fuji Film, Tokyo, Japan).

Nuclear run on assay

Cells were washed with ice-cold PBS, dislodged by rubber policeman and collected by centrifugation at 500 g for 5 min. The cell pellet was incubated in buffer I (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, and 0.4% Nonidet P-40, pH 7.4) on ice for 5 min and centrifuged at 500 g for 5 min. The nuclear pellet was washed with buffer I by centrifugation and suspended in buffer II (50 mM Tris-HCl, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA, pH 8.3). Nuclei (10^6/100 μl) were incubated at 30°C for 30 min with 100 μl of buffer III (10 mM Tris-HCl, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each ATP, CTP and GTP, and 200 μCi of [α-^{32}P]UTP), after which DNA was digested by RNase-free DNase at 30°C for 10 min. After treatment by 200 mg/ml proteinase K at 50°C for 1 h, newly transcribed RNAs were extracted by using Trizol. ^{32}P-labeled RNAs ($2x10^6$ cpm/ml) were hybridized overnight at 70°C in Rapid hybridization buffer (Amersham) with a nylon membrane spotted with 10 μg plasmid inserted full coding region of rat AM (154-711, D15069) or GAPDH (Clontech) in pBluescript SK+ digested with EcoRI. The membrane was sequentially washed with 2 x SSC containing 0.1% SDS at 65°C for 15 min, and 0.2 x SSC at 65°C for 1 h and subjected to autoradiography.

Results

Effects of NGF on AM and PAMP expression

We first examined how AM expression was modified by various growth factors. ir-AM level was significantly decreased by 35% and 20% by NGF and basic FGF, respectively, during 3 days of culture (Fig. 1). Insulin and PDGF slightly increased whereas EGF decreased AM expression, but they were not statistically significant. Since AM expression was most strongly modified by NGF, we focused to study the regulatory mechanism of AM and PAMP expression by NGF.

Expression of AM and PAMP were changed in the similar manner by the treatment of NGF. ir-AM and PAMP levels in the cells tended to increase slightly until 12 hours after the addition of NGF and then decreased to 60% and 75% of the control, respectively, during 3 days of culture (Fig. 2). Most of cells cultured with NGF became polygonal and extended more than two neurites during 3 days, whereas, in the absence of NGF, only a few cells bore short spine (Fig 3). Considerable

amount of ir-AM and ir-PAMP were released into the medium, and their changes were similar to those in the cells. It is noteworthy that ir-AM level in the medium was significantly increased by 30% 6 hours after the addition of NGF (Fig. 2 a, insert). ir-PAMP level also changed similar to ir-AM, but it was not a significant change (Fig. 2 c, insert). This may be due to rapid degradation of PAMP by neutral endopeptidase or due to the easily absorbable property of PAMP to plastic dish due to its positive charge.

Protein content in the cells cultured with NGF increased more than that in the control cells (0.754 + 0.082 mg/dish, n=6, and 1.005 + 0.075 mg/dish, n=5, in the control and NGF treated cells after 3 days of culture, respectively). Thus, the decreased level of AM by NGF during long term culture is not due to the loss of the cells during the incubation, but is due to the decreased expression of the peptides by NGF.

Effects of NGF on localization of AM and PAMP

To assess the change in the intracellular localization of AM and PAMP, cells were stained immunohistochamically. In the control cells, ir-AM and ir-PAMP were present in cytoplasm but not in nucleus (Fig. 3). Changes in ir-AM and ir-PAMP levels could not be detected 1-3 hours of incubation with NGF, but their intensity significantly decreased during 2 or 3 days of culture, in agreement with the decrease in ir-peptide levels measured by radioimmunoassay. There was no significant AM accumulation in the neuritis in comparison with in cytoplasm in the NGF treated cells.

Effects of NGF on mRNA level

mRNA encoding both AM and PAMP (referred as AM mRNA) was identified as a major band of 1.6 kb by northern blot (Fig. 4 a). mRNA level increased more than 3 fold 1 hour after the addition of NGF, then decreased progressively below the control level from 6 hours after the addition of NGF, and reduced to 10% of the control at 3 days of culture. mRNA level for GAPDH did not change significantly by NGF.

The degradation rate of AM mRNA measured by the addition of actinomycin D was rather slowed by NGF at 0.5 hour and 3 days after the addition of NGF. The slowed degradation rate even 3 days after the addition of NGF (when the mRNA level is decreased), indicates that the changes in AM mRNA level by NGF are mainly due to the changes in its transcription (Fig. 5).

Nuclear run on assay showed that the transcription rate of the peptide was

increased by 50% at the initial phase of incubation (0.5 hour), and then decreased to the undetectable level at 3 days after treatment with NGF (Fig. 6).

Discussion

In the present study, we have demonstrated that the expression of AM and PAMP is modified during neuronal differentiation. AM and PAMP levels are changed by NGF in the similar manner. mRNA level for these peptides changed biphasically after the addition of NGF: it increased transiently 1hour after the addition of NGF, and then decreased during neuronal differentiation by long-term culture. The transient increase in their mRNA level may be correlated with the increase in ir-AM and ir-PAMP accumulation in the medium shortly after the addition of NGF. The decrease in their expression during long-term culture is due to the suppression in their transcription, but not due to the acceleration of their mRNA degradation. The stability of their mRNA rather increased in the presence of NGF. These results indicate that neuronal differentiation suppresses the expression of AM and PAMP via the decrease in AM gene transcription, and that the suppression of AM and PAMP expression may play an important role in the development and maintenance of the nervous system.

<u>Regulation of AM and PAMP expression by extra- and intra-cellular signals and by cell differentiation</u>

Although regulatory mechanism of PAMP expression has been studied only in the cultured bovine adrenal chromaffin cells (Kobayashi et al. 1999), several data indicate that the AM expression in a variety of cell types is regulated by multiple extra- and intra-cellular signals.

In the vascular smooth muscle cells, growth factors (basic fibroblast growth factor, epidermal growth factor) and vasoactive substances (angiotensin II, endothelin 1) increased AM expression (Sugo et al. 1995a), but they decreased it in the vascular endothelial cells (Isumi et al. 1998). AM expression was strongly stimulated by tumor necrosis factor, interleukin-1 (IL-1) and lipopolysaccharide (LPS) (Sugo et al. 1995b), which are the major factors inducing septic shock, in the cultured vascular smooth muscle cells. However, AM expression in the endothelial cells was stimulated only small extent by these factors (Isumi et al. 1998). Furthermore, 8-bromo cyclic AMP or dbcAMP, a membrane permeable analogue of cyclic AMP,

decreased AM expression in the vascular smooth muscle cells and the cultured bovine adrenal chromaffin cells, but did not modify AM expression in the rat endothelial cells (Sugo et al. 1995a; Isumi et al. 1998).

The differences of the regulatory mechanisms of AM expression among various cell types indicate that extra- and intra-cellular signals do not directly regulate AM gene transcription, but rather cell differentiation is the major factor regulating AM expression. In fact, it has been shown that AM expression of several cell types is regulated by cell differentiation. In blood cells, differentiation from monocyte to macrophage increased AM expression more than 3 fold (Kubo et al. 1998). The AM expression in lymphocytes and granulocytes was 1/10 of macrophages (Kubo et al. 1998). In addition, phorbol ester and retinoic acid, differentiation and activation factors of monocyte increased AM secretion from RAW264.7 macrophage-like cells. In the case of blood cells, the differentiation toward macrophage increases AM expression. On the other hand, in the mouse 3T3-L1 cells, AM expression decreased during differentiation toward adipocytes (Li et al. 2003).

In the present study, AM and PAMP expression in PC12 cells decreased during neuronal differentiation by NGF. It is an interesting issue whether the decrease of their expression is cause or consequence of neuronal differentiation, but we have not observed a significant effect of AM and PAMP on cell differentiation when they were added to PC12 cells during culture (data not shown). In the rat adrenal medulla, AM expression was higher in phenylethanolamine N-methyltransferase (PNMT) positive adrenaline-secreting chromaffin cell than in PNMT negative noradrenaline-secreting cells (Renshaw et al. 2000). These results may indicate that the AM expression is high in the cells differentiated toward endocrine cells whereas low in the cells differentiated toward neuronal cells.

Regulation of peptide expression by NGF in PC12 cells

Expression of various peptides in PC12 cells is independently regulated by growth factors and hormones, whose mechanisms have not been fully understood. For example, peptide levels of neuropeptide Y, neurotensin and VGF are increased dramatically up to 100-fold over 1-6 days by NGF with concomitant increase in their mRNA levels (Levi et al. 1985; Kislauskis and Dobner 1990; Sabol and Higuchi 1990). Levels of chromogranin A and chromogranin B, the major acidic proteins in the chromaffin granules, were increased by NGF (Laslop and Tschernitz 1992) due to the increase in transcription of these genes and its mRNA level (Mahata et al. 1999; Mahapatra et al. 2000). These results suggest that transcription activity of these

peptides is the major determinant for their expression. This is also true in the case of the regulation of AM and PAMP expression in the present study, that is, the changes in transcription of AM gene is well correlated with the changes in their mRNA and peptide levels.

In the present study, AM mRNA stability was increased by NGF even when the mRNA level was decreased. This phenomenon seems to be paradoxical from point of view of the regulation of gene expression. However, recent evidence has shown that the regulation of mRNA decay of several genes is a major point of their expression in the nervous system (Malter 2001). Several RNA binding proteins that regulate RNA decay have already been identified (Guhaniyogi and Brewer 2001). In addition, a various kinds of developmental and environmental stimuli have shown to modify gene expression through the changes in mRNA stability. Accordingly, elucidation of regulatory mechanisms and the significance of the change in RNA stability may give us novel aspects of neuronal development.

Significance of the decrease in AM expression by neuronal differentiation

In the brain, AM is concentrated in the supraoptic, the paraventricular and the infundibular nuclei of hypothalamus, and its expression in other regions is maintained at low level (Ueta et al. 1995). AM in the brain may act as not only a neurotransmitter or nouromodulator but also an autocrie/paracrine regulator of cerebral microvessels and in choroid plexus. In fact, AM and its receptors coupled to cAMP generating system were expressed in the rat cerebral microvessels (Kobayashi et al. 2000a) and in choroid plexus (Kobayashi et al. 2001b). In addition, AM increased transendothelial electrical resistance, reduced endothelial permeability for low molecular weight substances, decreased endothelial fluid phase endocytosis and activates the P-glycoprotein efflux pump in cultured brain microvascular endothelial cells, indicating that AM elevates blood-brain barrier function (Kis et al. 2001; Kis et al. 2003).

The important feature of AM expression is its induction by ischemia/hypoxia (Wang et al. 1995; Kobayashi et al. 2003). AM dilatates cerebral arteries in vitro, and increases brain blood flow (Baskaya et al. 1995). Thus, the induction of AM by ischemia in neuron may have a neuroprotective function by increasing blood flow and blood-brain barrier function against injury occurred by ischemia and hypoxia.

On the other hand, AM and PAMP are the regulators of cell growth and apoptosis depending on cell type. AM stimulated DNA synthesis and cell proliferation of various cells including vascular endothelial cells, Swiss 3T3 fibroblast

cells (Withers et al. 1996) and tumor cells (Miller et al. 1996), whereas inhibits growth of mesangial cells (Michibata et al. 1998) and vascular smooth muscle cells (Kano et al. 1996). AM also prevents apoptosis of endothelial cells (Kato et al. 1997). In this line, the decrease in AM and PAMP expression during neuronal differentiation found in the present study may indicate that low levels of AM and PAMP in neuron may be an essential state for the induction of these peptides to act as neuroprotective factors, and/or to keep appropriate turnover of various cells such as vascular smooth muscle cells and endothelial cells in the nervous system.

Peptides contained in synaptic or chromaffin vesicles have function other than hormone or autocrine/paracrine regulator of cell function. Recent data have shown that chromogranin B is localized also in the nucleus and acts as a transcription factor (Yoo et al. 2002). In fact, chromogranin B modified transcription of many genes, and induced genes (zinc finger protein, MEF2C, hCRP2, abLIM) and suppressed genes (hcKrox, T3-receptor, troponin C, integrin) by chromagranin B were identified. On the other hand, chromogranin A and chromogranin B play an on/off switch role for secretory granule formation (Kim et al. 2001; Huh et al. 2003). It would be intriguing to know whether AM and PAMP have the functions in the regulation of gene transcription and in the formation of secretory granules. The regulatory mechanism of AM and PAMP expression during neuronal differentiation presented in this study may provide clue to understand physiological and pathological function of peptides in vesicle in the nervous system.

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Legends for figures

Fig. 1. Effects of various growth factors on AM expression in PC12 cells.
Cells were cultured in the absence or presence of NGF (50 ng/ml), EGF (10 ng/ml), bFGF (20 ng/ml), insulin (100 nM) or PDGF (1 ng/ml) for three days. Peptides of the cells were extracted with acetate, and ir-AM was assayed by radioimmunoassay. Data are the mean ± SEM of four samples. * $p<0.05$, ** $p<0.01$, compared with control.

Fig. 2. Effects of NGF on AM and PAMP levels in PC12 cells.
Cells were cultured in the presence (●) or absence (○) of NGF for periods indicated in the horizontal axis, and ir-AM (a, b) and ir-PAMP (c, d) levels in the medium (a, c) and in the cells (b, d) were measured by radioimmunoassay. Inserts: ir-AM and ir-PAMP levels in the medium at 6 hours of culture. Data are the mean ± SEM of three samples from a representative of five separate experiments. * $p<0.05$, compared with control.

Fig. 3. Changes in AM and PAMP localization in PC12 cells.
Cells cultured in the presence (●) or absence (○) of NGF for 3 days were immunohistochemically stained with antibody against AM or PAMP. Note that heavily staining of cytosol in control cells became less intense in the differentiated cells with NGF. Bar: 20 μm.

Fig. 4. Changes in AM mRNA by NGF in PC12 cells.
Total RNA (10 μg) prepared from the cells cultured in the presence (●) or absence (○) of NGF was separated by agarose electrophoresis, transferred to a nylon membrane and hybridized with ^{32}P-labeled rat AM or GAPDH cDNA fragment (a). The intensity of the bands was measured by Bio-Imaging analyzer, and the relative AM mRNA level in comparison with the control was indicated in panel b. The data are the representative of three separate experiments.

Fig. 5. Increase of AM mRNA stability by NGF in PC12 cells.
Cells cultured in the absence (○) or presence of NGF for 0.5 hour (●) or for 3 days(▲) were further incubated with actinomycin D (10 μg/ml) for period indicated. Total RNA was separated by electrophoresis, hybridized with ^{32}P-labeled AM probe

and the bands were detected by Bio-Imaging analyzer (a). The relative AM mRNA level was expressed as ratio to the value before the addition of actinomycin D (b). The data are the representative of four separate experiments.

Fig. 6. Changes in transcription rate of AM gene by NGF.

PC12 cells were cultured in the absence or presence of NGF for 0.5 hour or for 3 days, and their nuclei were separated. The genes being transcribing were labeled with [^{32}P]UTP, and were hybridized with cDNA for AM or GAPDH fixed on nitrocellulose membrane. The intensity of the dots was analyzed by Bio-Imaging analyzer.

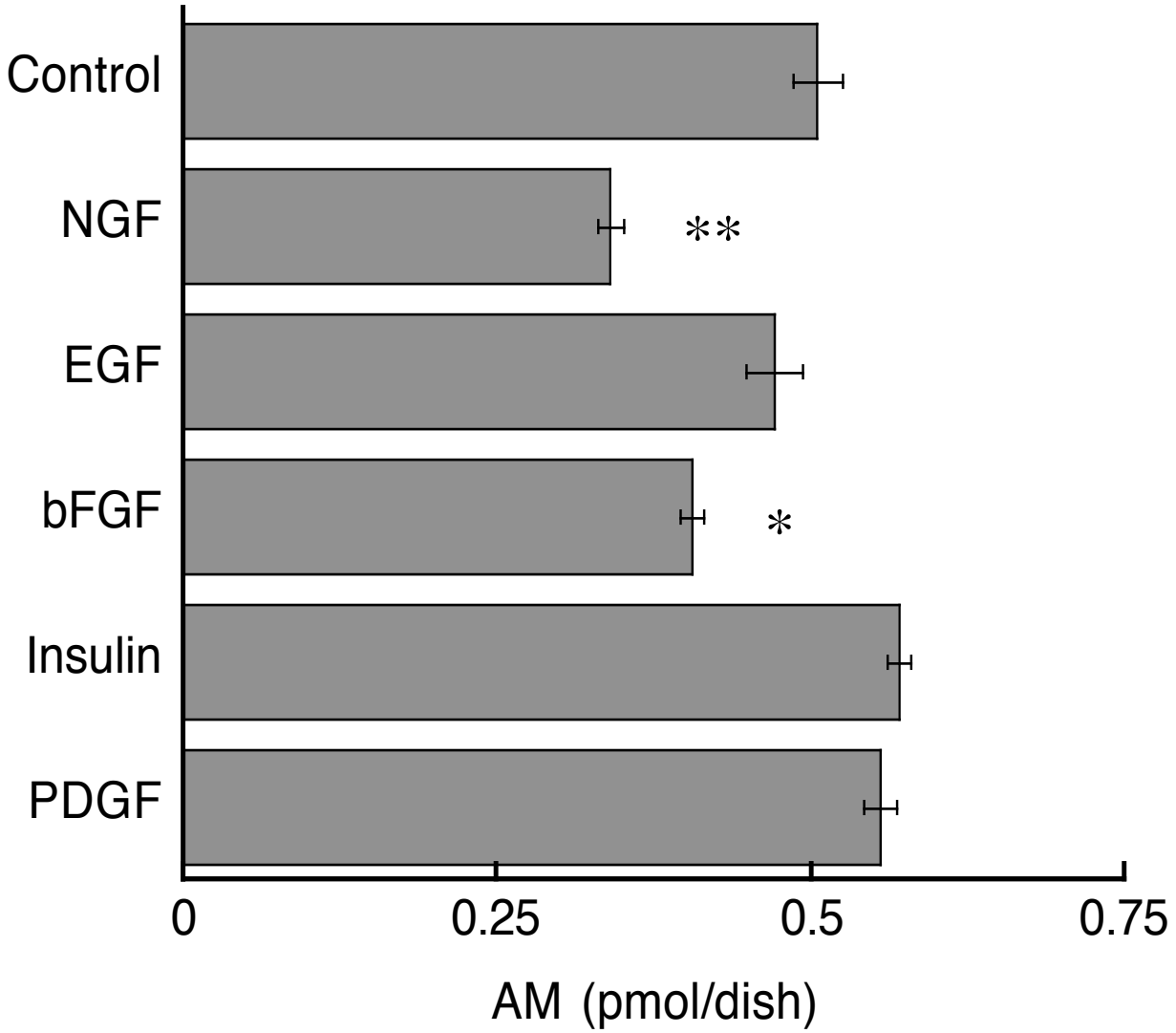

Fig. 1

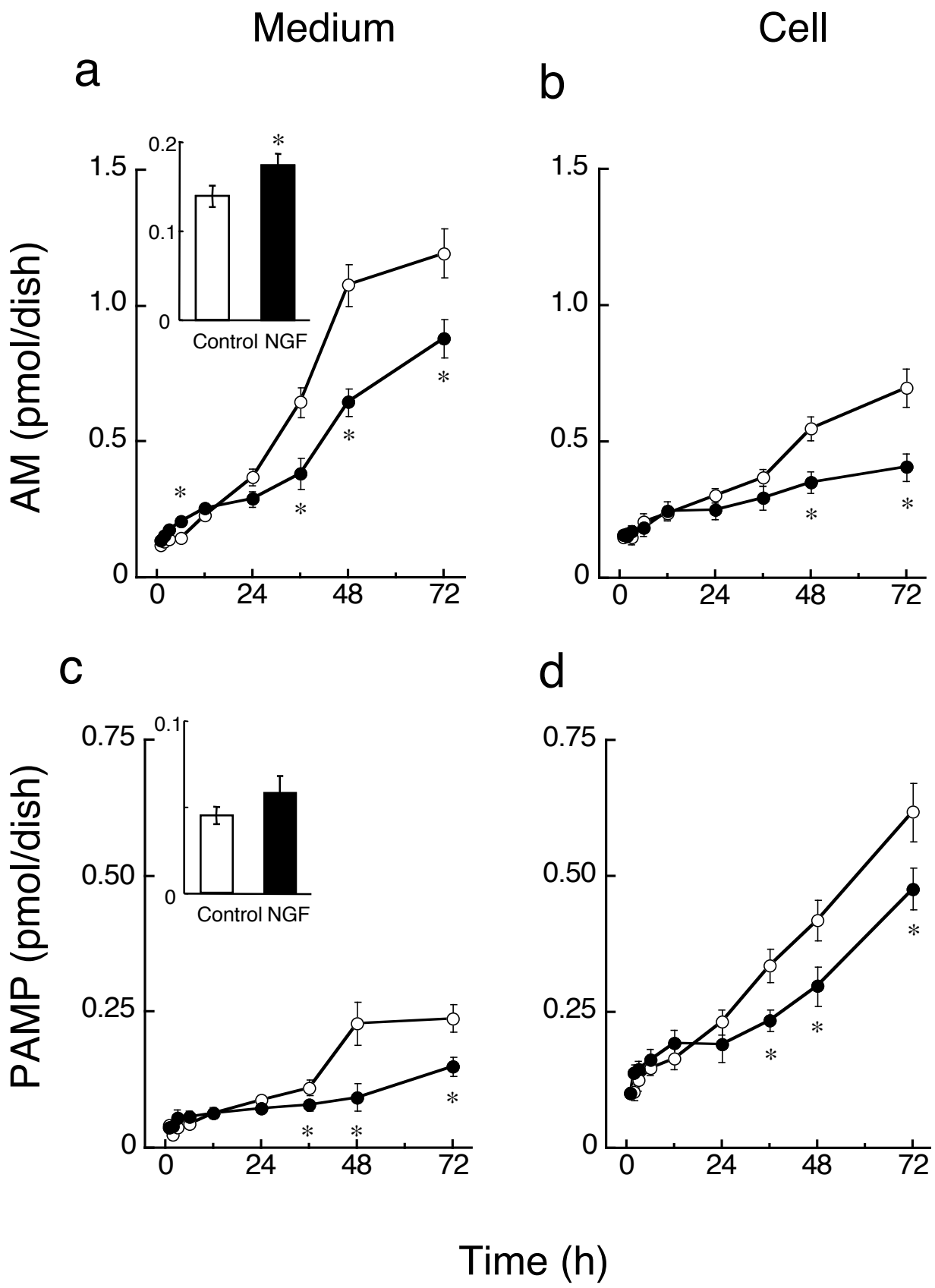
Medium
Cell
a
b
c
d
AM (pmol/dish)
PAMP (pmol/dish)
Time (h)
Control NGF
Control NGF
0
24
48
72
0.5
1.0
1.5
0.25
0.50
0.75
0.1
0.2
*

Fig. 2

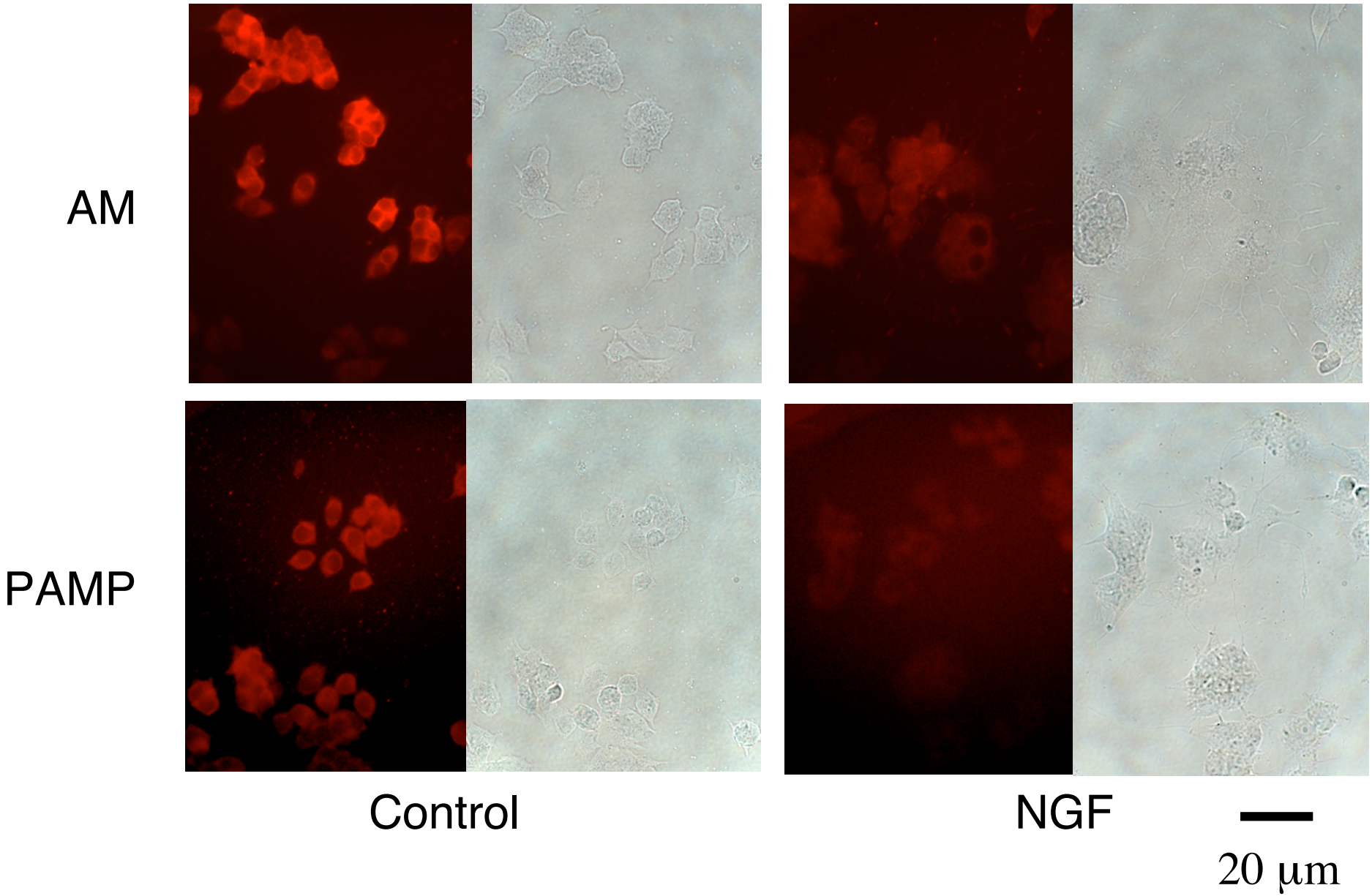

Fig. 3

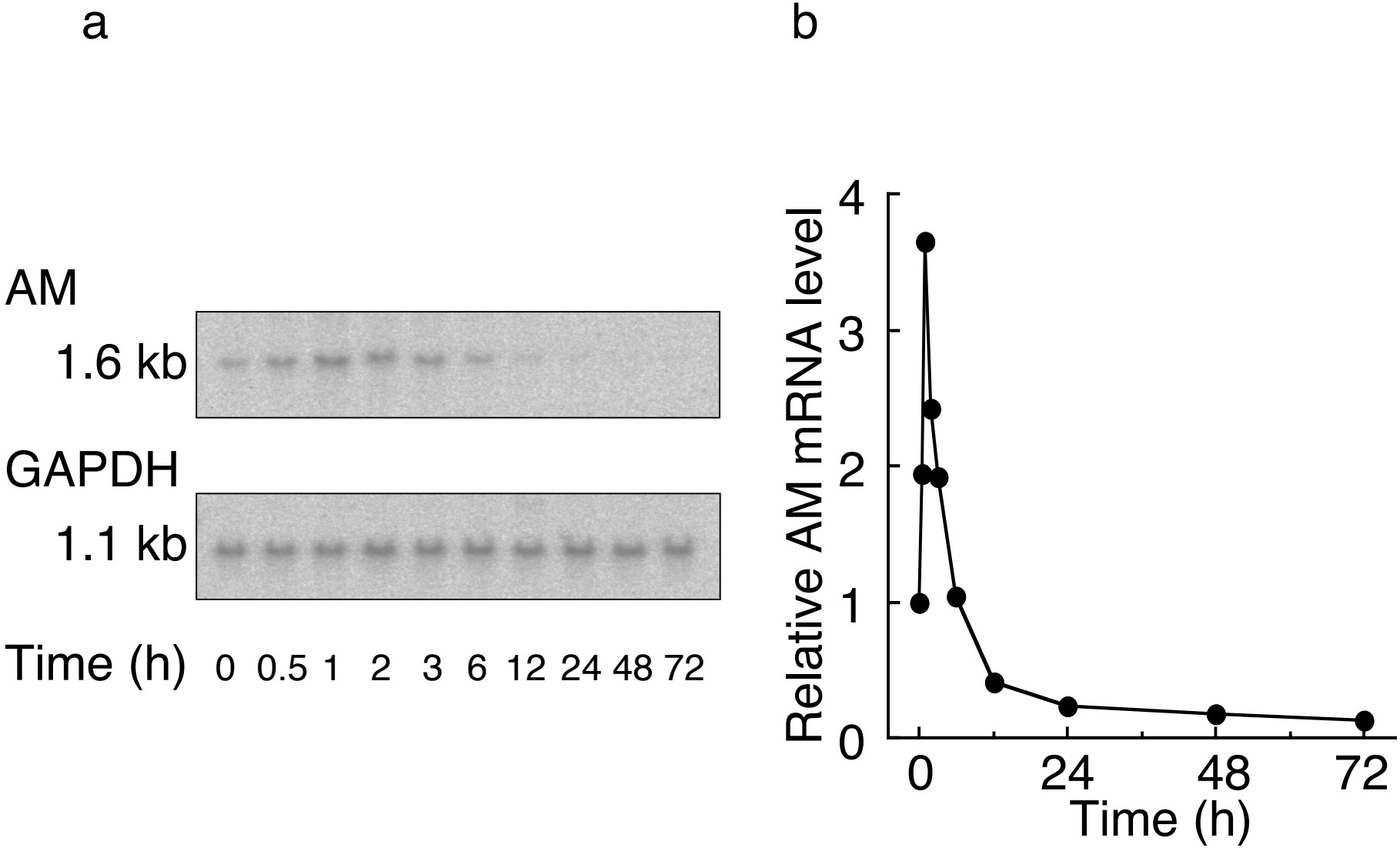
a
AM
1.6 kb
GAPDH
1.1 kb
Time (h) 0 0.5 1 2 3 6 12 24 48 72
b
Relative AM mRNA level
0
1
2
3
4
0
24
48
72
Time (h)

Fig. 4

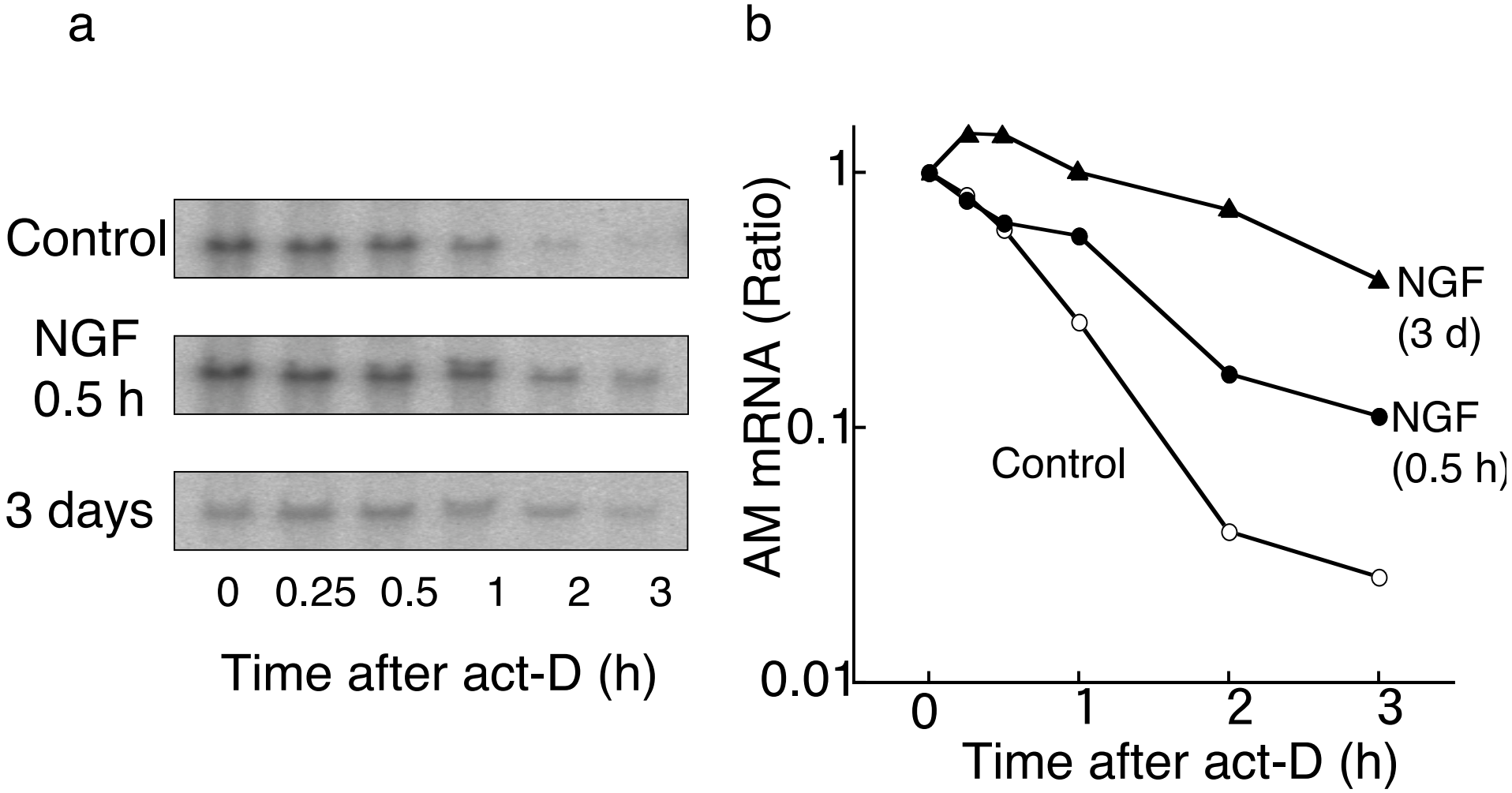
a
Control
NGF
0.5 h
3 days
0 0.25 0.5 1 2 3
Time after act-D (h)
b
AM mRNA (Ratio)
1
0.1
0.01
NGF
(3 d)
NGF
(0.5 h)
Control
0 1 2 3
Time after act-D (h)

Fig. 5

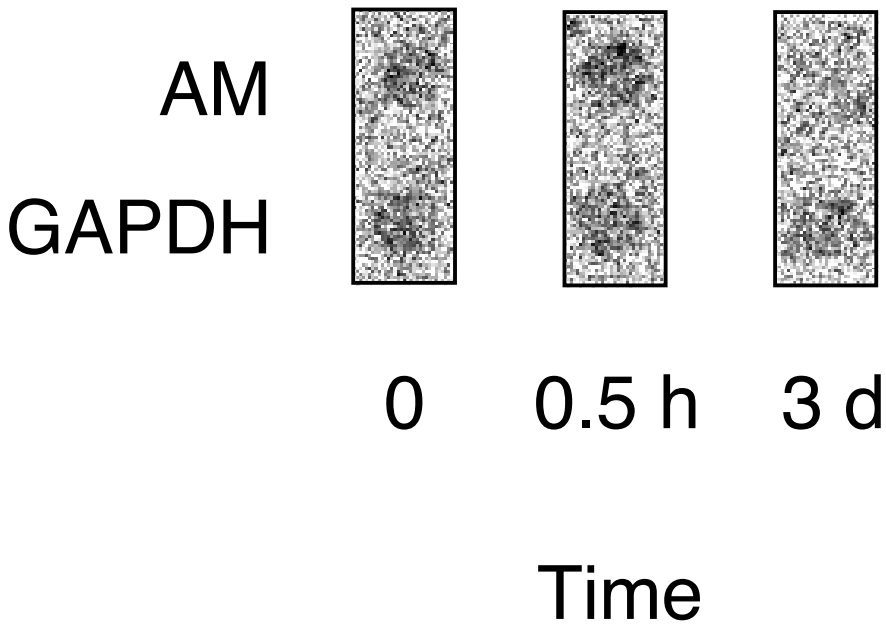

Fig. 6