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1-1-2010

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Recommended Citation

Rice KM, Walker EM, Kakarla SK, Paturi S, Wu M, Narula S. Blough ER (2010) Fluprostenol-induced MAPK Signaling is independent of aging in the Fischer 344/NNiaHSd X Brown Norway/BiNia Rat Aorta. Ann Clin Lab Sci 40:26-31.

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Fluprostenol-Induced MAPK Signaling Is Independent of Aging in Fischer 344/NNiaHSd x Brown Norway/BiNia Rat Aorta

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Abstract. The factors that regulate vascular mechanotransduction and how this process may be altered with aging are poorly understood and have not been widely studied. Recent data suggest that increased tissue loading can result in the release of prostaglandin F2 alpha (PGF2α) and other reports indicate that aging diminishes the ability of the aged aorta to activate mitogen activated protein kinase (MAPK) signaling in response to increased loading. Using ex vivo incubations, here we investigate whether aging affects the ability of the aorta to induce phosphorylation of extracellular signal-regulated kinase 1/2 (ERK½-MAPK), p38-MAPK, and Jun N-terminal kinase (JNK-MAPK) activation following stimulation with a PGF2α analog, fluprostenol. Compared to aortas from 6-mo animals, the amounts of ERK½- and p38-MAPK remained unchanged with aging, while the level of JNK-MAPK protein increased by 135% and 100% at 30- and 36-mo, respectively. Aging increased the basal phosphorylation of ERK½ (115% and 47%) and JNK (29% and 69%) ($p < 0.05$) in 30- and 36-mo aortas, while p38 phosphorylation levels remained unaltered. Compared to age-matched controls, fluprostenol induced phosphorylation of ERK½ (310%, 286%, and 554%), p38-MAPK (unchanged, 48%, and 148%), and JNK (78%, 88%, and 95%) in 6-, 30- and 36-mo aortas, respectively. These findings suggest that aging does not affect the ability of the rat aorta to activate ERK½-, p38-MAPK, and JNK-MAPK phosphorylation in response to PGF2α stimulation.

Keywords: aorta, fluprostenol, ERK½, p38-MAPK, JNK, aging

Introduction

Aging is the primary risk factor for cardiovascular disease (CVD). Although not well understood, it is thought that this increased risk may be due to age-associated changes in cardiovascular structure and function. Recently we reported that stretching of aortic vascular smooth muscle cells (VSMC) activates mitogen activated protein kinase (MAPK) signaling and that this response is altered with aging in Fischer 344/NNiaHSd x Brown Norway /BiNia rat (F344BN) aortas [1]. Specifically, we demonstrated that aging is associated with decreased ability of the aged aorta to activate p38-MAPK and c-Jun N-terminal kinase (JNK) signaling. The factors that regulate load-induced signaling in the aorta and other tissues are not well understood. In addition to mechanical stimuli, the possibility exists that autocrine and paracrine factors may play roles [2,3]. For example, Vandenburgh et al. [4] demonstrated that mechanical stimulation of cultured skeletal muscle increases the synthesis of prostaglandin F2 alpha (PGF2α) and that this increase is associated with the regulation of protein turnover and muscle cell growth. Similarly, it has

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0091-7370/10/0003-0026. $2.10. © 2010 by the Association of Clinical Scientists, Inc.

recently been shown that PGF2α can induce hypertrophy of neonatal rat ventricular myocytes and VSMC in vitro [5-8]. Whether the differences in mechanically-induced signaling that we observed in the aging F344BN aorta are due to alterations in the ability of the aged aorta to respond to PGF2α is unknown.

PGF2α receptor stimulation has been shown to induce MAPK activation in human endometrium epithelial cells [9] and cardiac myocytes [7]. The MAPK signaling network is composed of three homologous yet distinct kinase cascade pathways including the extracellular signal-regulated kinases (ERK)½, c-Jun NH_2-terminal kinase (JNK), and p38 kinase [10]. Studies in rats and humans have implicated the activation (phosphorylation) of MAPK signaling in the regulation of protein synthesis, mRNA stability, as mediators of apoptosis and the inflammatory response, and as key players in the control of load-induced alterations in protein expression [11-14]. Whether aging affects the ability of VSMC to activate MAPK signaling in response to PGF2α stimulation is not well understood.

Here we investigate if aging affects the ability of the aorta to respond to PGF2α by subjecting isolated aortas to a PGF2α analog, fluprostenol (FP). Our data demonstrate that fluprostenol activates ERK½, JNK, and p38-MAPK in aorta, and that fluprostenol-induced phosphorylation of MAPKs increases with aging. The data suggest that stretch-induced MAPK activation in the aging aorta is not associated with an inability of the aged aorta to respond to PGF2α.

Materials and Methods

Animals. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society and the Animal Use Review Board of Marshall University. All procedures were in accordance with the Public Health Service animal welfare policy. Adult (6-mo), aged (30-mo), and very aged (36-mo) male Fischer 344 /NNiaHSd x Brown Norway /BiNia (F344BN) rats were obtained from the National Institute on Aging (NIA) and were barrier-housed (2 rats/cage) in an AAALAC approved vivarium. Housing conditions consisted of 12 hr/12 hr dark-light cycle and a room temperature of 22 ± 2°C. The rats were provided food and water ad libitum. Rats were allowed to recover from shipment for at least two weeks before experimentation, during which time the rats were observed and weighed weekly. None of the older rats exhibited signs of failure to thrive, such as precipitous weight loss, disinterest in the environment, or unexpected gait alterations.

Materials. Antibodies against p38-MAPK (#9212), phospho-p38-MAPK (Thr180/Tyr182) (p-p38-MAPK, #9216), extracellular-regulated kinase 1/2 (ERK1/2, #9102), phospho-ERK1/2 (Thr202/Tyr204) (p-ERK1/2, #4377), c-Jun N-terminal kinase 1/2 (JNK1/2, #9252), phospho-JNK1/2 (Thr183/Tyr185) (p-JNK1/2, #9251), mouse IgG (#7076), and rabbit IgG (#7074) were from Cell Signaling Technology (Beverly, MA). Precast 7.5%, 10%, and 15% SDS-PAGE gels were from Cambrex Biosciences (Baltimore, MD). Enhanced chemiluminescent (ECL) western blot detection reagent was from Amersham Biosciences (Piscataway, NJ). Restore western blot stripping buffer was from Pierce (Rockford, IL). 3T3 cell lysates were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluprostenol and all other chemicals were from Sigma (St. Louis, MO).

Artery preparation and ex vivo incubations. Rats were anesthetized with a ketamine-xylazine (4:1) cocktail (50 mg/kg ip) and supplemented as necessary for reflexive response. In a sterile, aseptic environment, the ventral surface of the thorax was shaved and the superficial musculature was exposed by means of a transverse incision through the skin distal to the thoracic cavity. After midline laparotomy and perforation of the heart, the aorta was isolated and removed from the left ventricle to the renal arch. Isolated aortas were cleansed of connective tissue, transected at the subclavian artery and the diaphragmatic insertion, and cut longitudinally. The vessel segment was denuded and allowed to equilibrate in oxygenated Krebs-Ringer bicarbonate (KRB) buffer at 37°C for 1 hr. Vessels were incubated for 30 min with vehicle (ethanol) or 1 μM fluprostenol, on the basis of a previous finding that this concentration of fluprostenol is sufficient to induce intracellular signaling and myocyte hypertrophy [7]. Following incubations, vessels were collected and snap frozen in liquid nitrogen. All vessel handling was done in oxygenated KRB buffer maintained at 37°C.

Immunoblotting. Samples were pulverized with liquid nitrogen and lysed on ice for 30 min in T-PER (2 ml/g tissue) (Pierce, Rockford, IL) supplemented with 100 mM NaF, 1 mM Na_3VO_4, 2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepsatin. After lysis, homogenates were centrifuged 10 min at 10,000 rpm and the supernatants collected. Protein concentrations of the supernates were determined in triplicate using the Bradford method (Pierce, Rockford, IL) with bovine serum albumin as a standard. Samples were diluted to a concentration of 1.5 mg/ml in SDS loading buffer, boiled for 5 min, and 30 μg of protein was analyzed using SDS-PAGE. Transfer of protein onto nitrocellulose membranes was performed using standard conditions [9] and equal loading between lanes and membranes was verified by staining the membranes with Ponceau S. For immunodetection, membranes were blocked with a solution of 5% nonfat dry milk prepared in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hr at room

temperature and then incubated with the appropriate primary antibody overnight. After washing in TBST, the membranes were exposed to horseradish peroxidase-labeled (HRP) IgG secondary antibody (1 hr at room temperature).

Protein bands were visualized with ECL (Amersham Biosciences). Exposure time was adjusted to keep the integrated optical densities (IODs) within a linear and non-saturated range, and band signal intensity was quantified by densitometry using a flatbed scanner (Epson Pefection 3200 PHOTO) and imaging software (AlphaEaseFC). Molecular weight markers (Cell Signaling) were used as molecular mass standards and NIH 3T3 cell lysates were included as positive controls.

Data Analysis. Results are reported as mean ± SE. Data were analyzed using SigmaStat 3.0. Multiple group comparisons were performed by one-way ANOVA followed by Student-Newman-Keuls post hoc test to determine differences between groups. The level of significance accepted was $p < 0.05$.

Results

Aging effects on basal expression of JNK. To determine whether aging influences the total protein levels of ERK½-, p38-MAPK, and JNK present in the aorta, immunoblot analyses were performed using antibodies that recognize both the unphosphorylated and phosphorylated forms of the proteins. The Western blots failed to show any significant changes in the expression of ERK½ and total p38-MAPK with aging (Fig. 1). Compared to 6-mo animals, the amount of JNK was 135 ± 5% and 100 ± 6% higher in 30- and 36-mo aortas, respectively ($p < 0.05$).

Age-associated alterations of MAPK basal phosphorylation in aorta. Because ERK½, JNK, and p38-MAPK are thought to be regulated by phosphorylation, it was of interest to determine if the basal phosphorylation status of these proteins is altered with aging. When compared to 6-mo aortas, ERK½ phosphorylation was increased by 115 ± 5% and 47 ± 8% at 30- and 36-mo, respectively ($p < 0.05$) (Fig. 2). JNK phosphorylation was increased by 29 ± 9% and 70 ± 5% at 30- and 36-mo ($p < 0.05$) (Fig. 4). Conversely, basal phosphorylation of p38-MAPK was unaltered with aging (Fig. 3).

Fluprostenol-induced MAPK phosphorylation in aorta. PGF2α primarily mediates its cellular effects by binding with high affinity (K_i = 2.4 nM) to the FP prostanoid receptor [10]. To investigate the

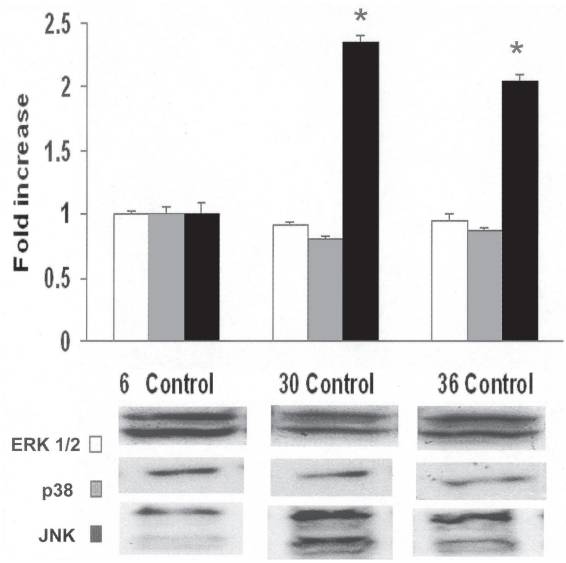

Fig. 1. Expression of ERK½, p38-MAPK, and JNK in aortas from 6-mo (young adult), 30-mo (aged) and 36-mo (very aged) Fischer 344/Brown Norway F1 hybrid rats. Relative changes in protein levels were determined by immunoblotting. The data are presented as fold-increase over the 6-mo value (n = 4). An asterisk indicates significant difference from the corresponding 6-mo value ($p < 0.05$).

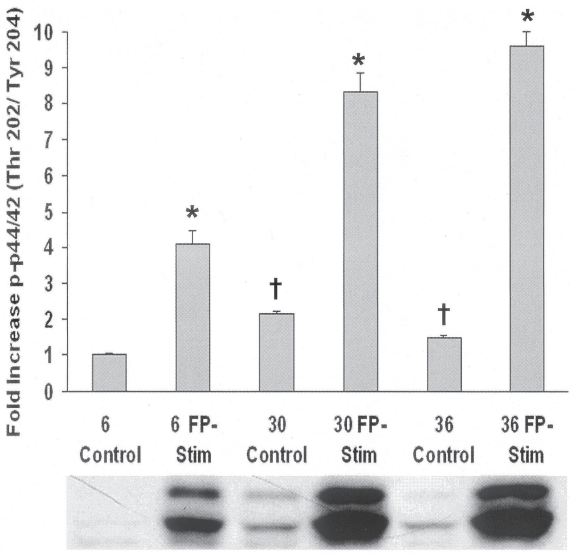

Fig. 2. Basal (control) and fluprostenol-induced phosphorylation of ERK½ in aortas from 6-, 30- and 36-mo old rats. Results were obtained by Western blot with immunodetection of ERK½ phosphorylated at Thr202/Tyr204. An asterisk or cross indicates significant difference from the control or the corresponding 6-mo value, respectively ($p < 0.05$), n = 4.

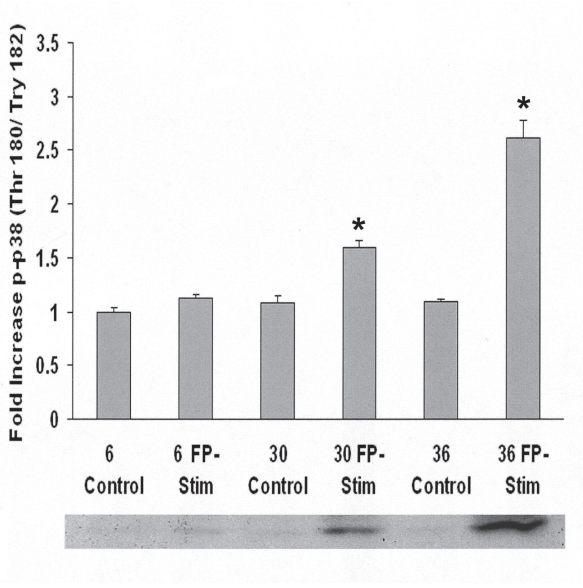

Fig. 3. Basal (control) and fluprostenol-induced phosphorylation of p38-MAPK in aortas from 6-, 30-, and 36-mo old rats. Results were obtained by Western blot with immunodetection of p38 phosphorylated at Thr180/Tyr182. An asterisk or cross indicates significant difference from the control or the corresponding 6-mo value, respectively ($p < 0.05$), n = 4.

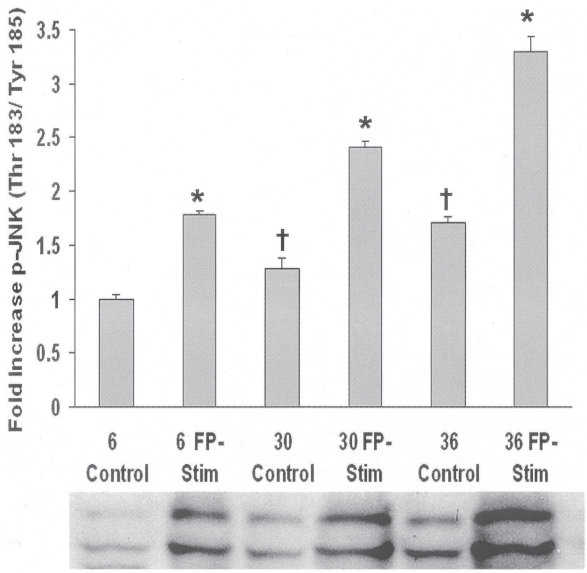

Fig. 4. Basal (control) and fluprostenol-induced phosphorylation of JNK-MAPK in aortas from 6-, 30- and 36-mo old rats. Results were obtained by Western blot with immunodetection of JNK phosphorylated at Thr183/Tyr185. An asterisk or cross indicates significant difference from the control or the corresponding 6-mo value, respectively ($p < 0.05$), n = 4.

signaling evoked by FP receptor activation we stimulated rat aortic smooth muscle with the PGF2α receptor agonist, fluprostenol. Fluprostenol was chosen as an agonist because it has a similar affinity for the fluprostenol receptor as PGF2α (K_i = 3.8 nM) but does not bind to other prostanoid receptors. As shown in Figs. 2, 3, and 4, incubation with fluprostenol increased phosphorylated levels of MAPKs. Compared to unstimulated controls, the phosphorylation of ERK½ was increased 310 ± 36%, 286 ± 51%, and 554 ± 38% at 6- , 30- and 36-mo, respectively ($p < 0.05$) (Fig. 2). Similarly, fluprostenol increased JNK phosphorylation by 78 ± 4%, 88 ± 6%, and 95 ± 14% at 6-, 30- and 36-mo, respectively ($p < 0.05$) (Fig. 4). Conversely, fluprostenol failed to affect p38-MAPKMAPK phosphorylation in 6-mo animals (Fig. 3), while in 30- and 36-mo animals the stimulation increased p38-MAPK phosphorylation by 48 ± 6% and 148 ± 16% ($p < 0.05$), respectively (Fig. 3).

Discussion

In adult tissues, the mechanisms regulating ERK½, p38-MAPK, and JNK activation by mechanical stress have been linked to stretch-induced release of nitric oxide (NO), arachidonic acid metabolites, and prostacyclin from the endothelial cells acting upon the VSMC [15-17] as well as PDGF, fibroblast growth factor, and ATP [18-23]. Previously we demonstrated that an acute elevation in loading pressure in vitro can increase the phosphorylation status of ERK½, p38-MAPK, and JNK in aortas of rats [1]. Importantly, we also demonstrated that aging reduced the ability of the aorta to activate p38-MAPK and JNK proteins. These data suggest that aging alters the ability of the aorta to "sense" and "respond" to mechanical stimuli.

One finding of the present study is that aging appears to increase JNK expression in aorta (Fig. 1). A similar increase in the amount of JNK-MAPK with aging was demonstrated previously in the aging F344BN aorta [24]. It is unclear why aging may increase the expression of JNK. It is thought that the JNK is a "stress kinase" [25,26]. Recent data suggest that aging may be associated with increases in the amount of reactive oxygen species (ROS) [24,27,28]. Whether this age-related increase

in ROS is related to changes in JNK expression is not known. JNK phosphorylates numerous proteins that reside in mitochondria or act within nuclei. This phosphorylation modifies the activity of these proteins allowing JNK activity to regulate several important cellular functions. Inflammatory signals, changes in levels of ROS, ultraviolet radiation, protein synthesis inhibitors, and a variety of stress stimuli have been shown to activate JNK.

Muscle stretch or loading has been shown to induce the release of PGF2α, which has a potential to activate MAPK proteins [29]. Whether aging affects the ability of the aorta to respond to PGF2α and whether this effect, if present, could help to explain differences in the ability of the aged aorta to "respond" to increases in loading is unknown. In the present study, we found that fluprostenol can activate MAPK proteins in the intact rat aorta (Figs. 2, 3, 4), which is consistent with previous data using cultured VSMC cells [30,31] and other work utilizing porcine carotid arteries [9].

The ability of fluprostenol-induced phosphorylation of MAPK proteins is retained in the aged F344BN rat aortas. Compared to 6-mo rats, FP-induced phosphorylation of both the ERK½ and JNK proteins in aged aorta was similar (Figs. 3, 4). Nonetheless, it should be noted that age-associated differences in the fluprostenol response appear to exist. Whereas fluprostenol stimulation fails to elicit phosphorylation of p38 in the 6-mo aortas, a marked increase in the amount of p38-MAPK phosphorylation was observed in 30- and 36-mo aortas (Fig. 2). The mechanism of this age-related difference in p38-MAPK activation is unknown.

In conclusion, our data suggest that aging does not diminish the ability of the aorta to activate MAPK proteins in response to fluprostenol stimulation. This finding is consistent with the possibility that age-related changes in the ability of the aorta to undergo MAPK activation in response to increased loading is not due to an inability of the aged aorta to respond to PGF2α. Nonetheless, it should be noted that it is not known if aging affects the amount of PGF2α generated upon aortic stretch. It is also unknown if aging alters PGF2α receptor density. Perhaps age-related decreases in PGF2α release or receptor density could help to explain why aortic mechanotransduction may be altered with aging. Further experiments will be useful in addressing these limitations.

Acknowledgement

This study was supported by National Institute on Aging Grant AG-20370 to Eric Blough.

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