It is hypothesized that differential AKT phosphorylation between sexes is important in abdominal aortic aneurysm (AAA) formation. Male C57BL/6 mice undergoing elastase treatment showed a typical AAA phenotype (80% over baseline, $P < 0.001$) and significantly increased phosphorylated AKT-308 (p308) and total-AKT (T-AKT) at day 14 compared with female mice. Elastase-treated Raw cells produced increased p308 and significant amounts of matrix metalloproteinase 9 (MMP-9), and these effects were suppressed by LY294002 treatment, a known AKT inhibitor. Male and female rat aortic smooth muscle cells treated with elastase for 1, 6, or 24 hours demonstrated that the p308/T-AKT and AKT-Ser-473/T-AKT ratios peaked at 6 hours and were significantly higher in the elastase-treated cells compared with controls. Similarly, male cells had higher phosphorylated AKT/T-AKT levels than female cells. LY294002 also inhibited elastase-induced p308 formation more in female smooth muscle cells than in males, and the corresponding cell media had less pro-MMP-9. AKT siRNA significantly decreased secretion of pro-MMP-9, as well as pro-MMP-2 and active MMP-2 from elastase-treated male rat aortic smooth muscle cells. IHC of male mice AAA aortas showed increased p308, AKT-Ser-473, and T-AKT compared with female mice. Aortas from male AAA patients had a significantly higher p308/T-AKT ratio than female AAA tissues. These data suggest that AKT phosphorylation is important in the upstream regulation of MMP activity, and that differential phosphorylation may be important in sex differences in AAA. (Am J Pathol 2014, 184: 148e158; http://dx.doi.org/10.1016/j.ajpath.2013.09.016)
were used in this study. The experiments were approved by the University of Michigan (Ann Arbor) Universal Committee on the Use and Care of Animals (number 8593).

Mice were anesthetized under 2% isoflurane inhalational anesthetic, a midline laparotomy was performed, and the abdominal aorta from just below the left renal vein to the bifurcation was isolated.21,22 The aortic diameter (AD) was measured with a Spot Insight Color Optical Camera (Diagnostic Instruments, Sterling Heights, MI) attached to an operating microscope (SMX-800; Nikon, Melville, NY) using NIS-Elements software version D.3.10 (Media Cybernetics, Rockville, MD). The aorta was then perfused for 5 minutes with porcine pancreatic elastase (specific activity 6.6 U/mg protein; E1250; Sigma, St. Louis, MO) at a concentration of 0.332 U/mL in 1 mL isotonic saline (experimental or control). After aortic perfusion, AD measurements were obtained. Saline- and elastase-perfused aortas were measured on 1, 3, and 14 days after perfusion (N = 5 or 6 per treatment group per day). Segments of the infrarenal aorta were studied for Western blot analysis, immunohistochemistry (IHC), and zymography. AD increases were reported as a percentage increase from baseline measurements, and an AAA was defined as a 50% increase in AD compared with baseline.

To investigate whether inhibition of phosphorylation of AKT with LY294002 could prevent the formation of AAA in the elastase model, a separate set of experiments was performed, in which mice were divided into four groups, each with N = 8 mice. The groups of mice were as follows: i) mice only treated with dimethyl sulfoxide (DMSO) and LY294002 at a dose of 3 μg/g body weight of mice (No Surgery group), ii) mice that received elastase perfusion as described before and also received DMSO treatment (Surgery + DMSO group but no LY294002), iii) mice that received elastase perfusion as described before and treated with LY294002 and DMSO (Surgery + DMSO + LY group), and iv) mice that underwent elastase perfusion as described earlier (Surgery only group) but received no LY294002 or DMSO treatment. Treatment of mice consisted of i.p. injection of LY294002 (dissolved in 25% DMSO in PBS) at a dose of 3 μg/g body weight of mice every day from day 4 after elastase perfusion to day 14 (the day the mice were sacrificed and tissue was harvested). DMSO treatment consisted of i.p. injection of 25% DMSO in PBS from day 4 after elastase perfusion to day 14 to investigate whether DMSO alone had any effect on AAA formation.

Cell Culture

Male and female RASMCs were isolated, as described previously,7 and were cultured in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen) and 1% penicillin, streptomycin, and glutamine mixture (Invitrogen) at 37°C in a humidified, 5% carbon dioxide atmosphere. Cells (passages 5 to 8) were treated with elastase at 5 μg/mL for 24 hours in serum-free Dulbecco’s modified Eagle’s medium containing the antibiotics and 1% bovine serum albumin. For the in vitro inhibition studies, the cells were pretreated for 2 hours with 50 μmol/L LY294002, an inhibitor of AKT phosphorylation, before treatment with elastase. Media were collected at 1, 6, and 24 hours and assayed for MMP-2 and MMP-9 activities by zymography. RASMCs were lysed, and total AKT (T-AKT), phosphorylated AKT-Thr-308 (p308), and AKT-Ser-473 (p473) levels were assessed by using Western blot analysis (N = 3 per time point per sample, repeated in triplicate).

siRNA Transfection

Cells were plated in 6-well tissue-culture plates at 60% to 80% confluency. One day later, the cells were transfected with 1 μmol/L of siRNA per well for AKT1 (Thermo Scientific, Pittsburgh, PA) or 200 pmol per well for control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using the Accell siRNA delivery medium for AKT siRNA (Thermo Scientific). For control siRNA, siRNA transfection reagent and medium from Santa Cruz Biotechnology, according to the manufacturer’s instructions, were used. Seventy-two hours after transfection, cells were washed with PBS, starved in serum-free medium overnight, washed in PBS, and then treated with elastase-containing media or media alone for a further 24 hours. The media and cells were then collected for zymography and Western blot analysis, respectively.

Western Blot Analysis

Proteins were isolated from aortic segments (N = 3 aortas per time point per sample, repeated in triplicate) using radioimmunoprecipitation assay buffer (Thermo Scientific), containing 1% SDS by sonication, and overnight incubation on a shaker. Protein concentration was measured by a bicinchoninic acid kit (Thermo Scientific), and equal amounts of proteins were loaded in each lane for gel electrophoresis using 10% Bis-Tris Nupage gels (Invitrogen). The electrophoretically separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA) by using a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, CA), as per the manufacturer’s guidelines. Non-specific binding was blocked by incubating the membrane for 1 hour in StartBlock TBS (Thermo Scientific). The membranes were incubated with primary antibodies in blocking buffer at 4°C overnight, washed with 25 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween-20, pH 7.4, and then incubated with horseradish peroxidase–conjugated secondary antibodies in blocking buffer for 1 hour, washed in 25 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween-20, pH 7.4, and developed with the West-Pico electrochemiluminescence kit (Thermo Scientific). The primary antibodies used were phosphorylated Akt (Ser473) (D9E) XP Rabbit monoclonal antibody (mAb) 4060, lot 9, phosphorylated Akt (Thr308) (C31E5E) Rabbit mAb 2965, lot 3, Akt (pan) (C67E7) Rabbit mAb 4691, lot 11 (all from Cell Signaling Technology, Danvers, MA; dilution 1:500),
**Substrate Gel Zymography**

Gelatin substrate zymograms were run in precast 10% SDS-PAGE gels containing 1 mg/mL of gelatin (Invitrogen). Equal volumes of experimental media or equal amounts of proteins were diluted into 2× Tris-glycine SDS sample buffer and electrophoretically separated under nonreducing conditions. Proteins were renatured for 30 minutes in Renaturing Buffer (Invitrogen) and then the gels were incubated in the Developing Buffer (Invitrogen) for 30 minutes and again in the same buffer overnight at 37°C. The gel was stained in SimplyBlue SafeStain (Invitrogen), and the gelatinase activity was observed by clear bands against the blue background.

**IHC Data**

Harvested aortas were fixed in fresh 4% paraformaldehyde for 16 to 24 hours, followed by 70% ethanol. Segments were then paraffin embedded, and sections (5 μm thick) were mounted onto slides. The sections were deparaffinized and rehydrated according to standard protocols, and those prepared for IHC studies were treated for 10 minutes with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were blocked and then incubated with the primary antibodies overnight at a dilution of 1:100 in blocking solution and stained according to the instructions in the Vectastain ABC Elite Kit (Vector Laboratories). The immunolocalized proteins were then visualized using the Novared kit (Vector Laboratories, Burlingame, CA), and anti-rat Texas Red TI-9400, lot W0322 (dilution 1:200) (Santa Cruz Biotechnology), and anti-rabbit secondary antibodies (Santa Cruz Biotechnology, dilution 1:200) (Cedarlane Laboratories Limited, Burlington, ON, Canada), anti-rabbit fluorescein isothiocyanate sc-2012, lot D1009 (dilution 1:200) (Santa Cruz Biotechnology, and anti-rat Texas Red TI-9400, lot W0322 (dilution 1:200) (Vector Laboratories, Burlingame, CA), were also used. β-Actin staining was used to check for equal loading of proteins.

**Data Analysis**

Data are expressed as means ± SE. The means of two groups were compared using a Student’s t-test. P < 0.05 was considered statistically significant.

**Results**

Increased p308 in Mouse AAA Model

The mouse AAA model was used to evaluate the status of AKT phosphorylation on different days (1, 3, and 14).7,23,24 Western blot data showed that on day 1 after elastase perfusion, there was no difference in the levels of p308 (P = 0.43) and T-AKT (P = 0.35) between male and female mice (Figure 1A). However, by day 3 after elastase infusion, there was a significant increase in the level of p308 (P = 0.0002) in male compared with female mice (Figure 1, A and B), although female mice had 147% more T-AKT than male mice (Figure 1, A and C). The level of p308 and T-AKT in male mice at 14 days after elastase infusion was significantly more (P = 0.0009 and P = 0.0162, respectively) than in female mice (Figure 1, D and E). The level of p473 in elastase-treated mice at all time points did not show any specific bands (data not shown). Increased phosphorylation of AKT in the elastase-perfused aorta in male mice correlated with increased AAA formation in male compared with female mice (Figure 2).

Increased Phosphorylation of AKT in Human AAA

Human tissues from AAA and control aortas were analyzed for their AKT content by using Western blot analysis...
Effect of Inhibiting AKT Phosphorylation on AAA Formation in Mice

Western blot experiments demonstrate that formation of AAA in mice and humans was accompanied with an increase in phosphorylation of AKT. We, therefore, wanted to investigate whether inhibiting AKT phosphorylation in vivo would have any effect on AAA formation in mice. We injected mice with LY294002 (an inhibitor of AKT phosphorylation) at only a single dose of 3 μg/g, before subjecting the mice to elastase perfusion, as described earlier. We observed that treatment with LY294002 after elastase perfusion did not completely inhibit AAA formation (decrease of 5.5%) (data not shown). However, Verhoff’s–van Gieson staining, as well as H&E counterstaining, showed that the elastin fibers were less disrupted in the LY294002-treated mice compared with those mice that received DMSO (used to dissolve LY294002) treatment or no drug treatments (surgery only) (Figure 4).

We next performed IHC to visualize the presence of p308, p473, and T-AKT in male mice that were treated with LY294002 after perfusing their aorta with elastase. As seen in Figure 5, mice that were treated with LY294002 after elastase perfusion (surgery + DMSO + LY) exhibited reduced staining for p308 and p473 compared with the mice that received no drug treatments (surgery only and surgery + DMSO groups). The staining for T-AKT between these groups of mice did not show any discernible differences. We also observed less macrophage infiltration in the LY294002-treated mice than those mice that did not receive the drug treatment, as demonstrated by less Mac-2 staining, a known marker for macrophages (Figure 5). Our IHC data were corroborated with Western blot experiments in which we observed significant differences in p308 and p308/T-AKT levels in the aortas isolated from the LY294002-treated mice compared with the DMSO + surgery and surgery-only groups of mice (Supplemental Figure S1, B and D). Moreover, Western blot data showed no differences in the levels of T-AKT between the different treatment groups of mice (Supplemental Figure S1C).

Immunofluorescence Analysis of p308 and Macrophages in Human and Murine AAA Tissues

Western blot data showed that, in both mice and humans, there was an up-regulation of p308 in the AAA tissues. It is known that macrophage infiltration occurs at the site of AAA formation.24 We, therefore, used immunofluorescence to localize p308 and Mac-2 staining in the aorta of the mice that were either treated or not treated with LY294002 after elastase perfusion. As seen in Figure 6, both in the drug-treated and untreated groups, there were areas of colocalization of p308-producing cells and Mac-2 cells, as seen in the merged immunofluorescence, which exhibited the characteristic yellow fluorescence. However, the LY294002-treated mice showed less yellow fluorescence, indicating less p308 production and macrophage infiltration in the tissue.

We performed similar experiments on tissues from human patients with AAA and compared p308 and Mac-2 staining with those of the control tissues. We observed similar patterns of colocalization of p308 and macrophages in human AAA tissues (Figure 7) as in mouse experiments. Moreover, colocalization was observed in both male and female AAA patients. In control human tissues, we did not observe colocalization of p308-producing cells and macrophages.

Figure 2 Increase in aortic diameter in male (circles) versus female (triangles) mice at days 1, 3, and 14. P < 0.001. WT, wild type.
Elastase-Induced p308 Formation in RAW Cells Is Attenuated by LY294002 Treatment

Immunofluorescence studies showed that p308-producing cells and macrophages were colocalized (Figures 6 and 7) in the AAA tissues. We, therefore, used in vitro cell culture assays to determine whether mouse macrophages were capable of producing p308 on stimulation with elastase.

Elastase, 5 μg/mL, was used for treating the cells. The dose is nontoxic and was chosen based on published literature. As seen in the Western blot analysis in Figure 8A, when male mouse macrophages (RAW 264.7 cells) were incubated for 24 hours with elastase, an agent used to induce AAA in our mouse model, p308, was increased (Figure 8A) compared with media-treated control cells. This p308 induction was subsequently abolished when the cells were pretreated with LY294002 that was dissolved in DMSO (surgery + DMSO + LY). Arrows show sites of intact and broken elastin fibers.
LY294002 (Figure 8A). Treatment of macrophages with elastase for shorter periods of 6 hours (Figure 8A) did not elicit p308 formation. Western blot analysis also showed that elastase treatment did not alter T-AKT and actin levels in these macrophages.

We next investigated whether these macrophages were capable of secreting MMPs when stimulated with elastase. As shown by zymography in Figure 8B and densitometric analysis in Figure 8C, elastase induced significant amounts of MMP-9 secretion from macrophages under in vitro conditions. However, when the macrophages were pre-treated with LY294002, an inhibitor for AKT phosphorylation, there was a significant decrease in MMP-9 secretion from the macrophages in the presence of elastase.

Elastase-Induced Activation of AKT in Male and Female RASMCs

Smooth muscle cells (SMCs) are the most abundant cell type in the aorta. Our immunofluorescence experiments with mouse and human tissues and a subsequent in vitro experiment with mouse macrophages had shown that the macrophages were able to produce p308 and secrete MMP-9 (Figures 6, 7, and 8). Therefore, in conjunction with macrophages, we wanted to investigate AKT phosphorylation and MMP-2 and MMP-9 production by aortic smooth muscle cells when they were treated with elastase and also to observe sex-dependent AKT phosphorylation in the SMCs in response to elastase.23 We observed a temporal response to elastase in that there was an increase in p308 and p473 production in both male and female RASMCs, which peaked at 6 hours (Supplemental Figure S2). There was a significant increase in production of p308 (P < 0.0001) (Supplemental Figures S3, A and B, and S4A) and p473 (P < 0.002) (Supplemental Figures S3, C and D, and S4C) at 6 and 24 hours. At all time points, male RASMCs had more p308 and p473 than female RASMCs (Table 1).

Pretreatment of RASMCs with LY294002 Decreases Elastase-Induced p308 and p473

LY294002 is a known inhibitor of AKT phosphorylation.30 Therefore, to confirm the phosphorylation of AKT in RASMCs in the presence of elastase, we pretreated RASMCs with LY294002 for 2 hours and then treated the cells with elastase in the presence of the inhibitor. As seen in the Western blot in Supplemental Figure S2, there was a significant reduction in p308 and p473 in female RASMCs compared with male RASMCs at 6 hours (Table 2).

Elastase Induced Higher Pro—MMP-2 Activities in RASMCs and Attenuation of Pro—MMP-9 Induction by AKT Inhibitor

To determine whether inhibiting AKT phosphorylation had any effect on MMP-2 and MMP-9 activity, zymography was performed on the media from the cells treated with either elastase or elastase in the presence of LY294002. Zymography showed that male RASMCs had higher levels of pro—MMP-2 than female RASMCs at all time points after elastase treatment (Figure 9 and Supplemental Table S1). Moreover, pro—MMP-9 activity at 24 hours was completely attenuated in female RASMCs by the AKT inhibitor, LY294002, compared with the male RASMCs (Figure 9).
AKT siRNA-transfected male RASMCs (Figure 10A). Densitometric analysis of the zymogram in the presence of elastase than control siRNA-transfected (Elast), and cells treated for 24 hours with elastase and LY294002 (Elast + Inh), cells treated with elastase for 24 hours. We, thus, transfected male and female RASMCs with siRNA for a target molecule in a non-specific manner. We, thus, transfected male and female RASMCs with siRNA for AKT and then treated them with elastase for 24 hours. We observed that AKT siRNA-transfected male RASMCs secreted less pro—MMP-9, pro—MMP-2, and active MMP-2 in the presence of elastase than control siRNA-transfected cells (Figure 10A). Densitometric analysis of the zymogram revealed that AKT siRNA-transfected male RASMCs secreted significantly reduced pro—MMP-9 (E versus AKT-si + E; \( P = 0.0089 \)) (Figure 10B), pro—MMP-2 (E versus AKT-si + E; \( P = 0.0097 \)) (Figure 10C), and active MMP-2 (E versus AKT-si + E; \( P = 0.0020 \)) (Figure 10D). Transfection of female RASMCs with AKT-siRNA did not have any effect on MMP production in the presence of elastase (data not shown).

### Discussion

AKT, a serine/threonine kinase, was first discovered in 1977 as an oncogene in the transforming retrovirus AKT8.\(^3\) It is a critical mediator of extracellular information into intra-cellular biological responses. AKT regulates a vast array of cellular responses, such as cell motility, growth, proliferation, apoptosis, survival, transcription, protein synthesis, and nutrient metabolism. There are three different AKT isoforms (AKT1, AKT2, and AKT3), all of which have similar structural homologies.\(^2\) AKT-mutant mice have shown that mutations in AKT1 may cause schizophrenia.\(^3\) AKT2 alterations may result in familial diabetes,\(^4\) whereas changes in AKT3 result in reduced size of the brain.\(^5\) AKT has been shown to be highly activated by low concentrations of insulin in female intra-abdominal adipose tissue compared with males.\(^6\) Cortical phosphorylated AKT was increased by isoflurane in male mice compared with female mice, and this resulted in better neuroprotection in the male mice.\(^7\) Again, hearts from female mice had greater recovery of left ventricular function and higher phosphorylated AKT levels compared with males.\(^8\) There are reports showing that young women have higher levels of nuclear-localized phosphorylated AKT in cardiomyocytes than comparably aged men or postmenopausal women.\(^9\)

Studies have shown that the hormones, estrogen and testosterone, mediate some of the effects of the AKT. In male C57BL/6J mice, estrogen confers cardioprotection after myocardial infarction by decreasing MMP-9 activity and increasing the AKT signaling pathway.\(^10\) Administration of

### Table 1

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>( p308/T\text{-AKT} P ) value (male vs female)</th>
<th>( p473/T\text{-AKT} P ) value (male vs female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>6</td>
<td>0.022</td>
<td>0.030</td>
</tr>
<tr>
<td>24</td>
<td>0.002</td>
<td>0.010</td>
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</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>( P ) value (male)</th>
<th>( P ) value (female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p308/T\text{-AKT} ) ratio</td>
<td>0.016</td>
<td>0.002</td>
</tr>
<tr>
<td>( p473/T\text{-AKT} ) ratio</td>
<td>0.117</td>
<td>0.004</td>
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testosterone, on the other hand, down-regulated the AKT pathway in male rat hearts after ischemia/reperfusion.\textsuperscript{40} Estrogen also activates the AKT pathway and induces vascular endothelial growth factor-A expression in the epithelial cells of the rat uterus.\textsuperscript{41} Estrogen also induces endothelial nitric oxide synthase via an AKT-dependent pathway in vascular endothelial cells.\textsuperscript{42} All of these studies suggest that AKT might play a role in sex-mediated disease differences.

By using the elastase perfusion AAA model, it was shown that male rats developed larger AAAs than female rats in association with increased macrophage infiltration and MMP-9 RNA expression in male rats compared with female rats.\textsuperscript{23} It was also shown that female rat aortas transplanted into male rats lost their resistance toward aneurysm formation and were similar to their male counterparts. Treatment with 17-\textbeta-estradiol reduced the growth of aneurysm, elastin disintegration, macrophage infiltration, and MMP-9 levels in male mice. By using the same elastase perfusion model, it was demonstrated that oophorectomized female rats exhibited an increased size of AAAs compared with oophorectomized female and male rats that received 17-\textbeta-estradiol treatment.\textsuperscript{43} This increased aneurysm formation correlated with increased levels of MMP-2 and MMP-9 expression in these oophorectomized and 17-\textbeta-estradiol-treated male and female mice.\textsuperscript{44} Moreover, in earlier research articles, we showed that male mice have more JNK-1 than female mice, which could account for a higher incidence of AAA in males.\textsuperscript{45} We also have shown that female mice have higher amounts of PAI-1 than male mice and, thus, PAI-1 could impart resistance to AAA formation in female mice.\textsuperscript{46} In another set of \textit{in vitro} studies, we have shown that \textit{p}-ERK signaling is up-regulated in male rat aortic smooth muscle cells.\textsuperscript{7} We have also shown that treatment of male rats with 17-\textbeta-estradiol greatly reduced the male aortic MMP-2 production.\textsuperscript{47} Our studies have also documented that there is decreased expression of multiple cytokines, chemokines, and leukocyte infiltration in female rat aortas compared with male rats in the elastase perfusion model of AAA.\textsuperscript{1} Therefore, from all of these studies, we conclude that female hormones regulate certain cytokines, chemokines, and proteins, such as JNK and ERK, having a protective role in aneurysm formation. The present study suggests that the sex-specific differences in AKT phosphorylation and MMP-2 and MMP-9 secretions that we observed in RASMCs in response to elastase treatment may be another component of the complex regulatory mechanisms that contribute to the differences that are observed in male and female animal models of AAA, as well as probably in humans.

AKT is a known modulator of MMPs.\textsuperscript{32} Tumor necrosis factor-\textalpha induces MMP-9 expression via activation of the AKT pathway in vascular smooth muscle cells and in mouse epidermal cells.\textsuperscript{11,12,48} The migration of vascular smooth muscle cells requires phosphorylation of AKT for the activation of MMP-2 and MMP-9.\textsuperscript{49} Cigarette smoke extract has been shown to induce MMP-9 expression in rat alveolar macrophages via phosphorylation of AKT.\textsuperscript{50} IL-1-induced MMP-1 and MMP-13 expressions in human chondrocytes were blocked by LY294002, suggesting that activation of AKT is important in cartilage collagenolysis.\textsuperscript{51}

Recently, it was demonstrated that AKT2 confers protection against aortic aneurysms and dissection.\textsuperscript{52} Using AKT2 knockout mice and infusing them with angiotensin II resulted in the knockout mice forming larger aortic aneurysms compared with the wild-type control. The said study also demonstrated that there is reduced expression of AKT2 in the degenerative medial layer of human aortic aneurysm dissection tissues. The angiotensin model of mice that was used is different from the elastase model of AAA disease in mice. In the elastase model, we observe aneurysms by day 14, whereas in the angiotensin II model, the mice develop aneurysm at approximately 28 days, with a more profound inflammatory response. Therefore, the intracellular events in the angiotensin and elastase model of AAA may not be comparable. Moreover, the authors did not look into the expression levels of AKT1 and AKT3 in their study to see whether the expression levels of these AKT isoforms differed in the AKT2 knockout mice that underwent angiotensin II treatment and also in the human AAA tissues. We agree with the authors of the previous studies that the literature documents that a link between AKT isoforms and inflammation is complex and often one isoform compensates for the other.

The present study is the first to implicate the importance of AKT during AAA formation in the elastase model of the disease in mice. In the present study, AKT phosphorylation was increased in males compared with females, both in rodents and in humans. In addition, we document that

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**Figure 9** Zymogram shows that elastase induces higher pro-MMP-2 activities in male RASMCs than female RASMCs. In addition, inhibition of AKT phosphorylation attenuated pro-MMP-9 induction at 24 hours in both male and female RASMCs.
elastase treatment of the aortic smooth muscle cells differentially turns on the AKT signaling pathway in the male and female RASMCs. These cells showed increases in phosphorylated AKT levels with increasing incubation times with elastase, which peaked at 6 hours. Male SMCs were more susceptible to increased phosphorylation of AKT than female cells. Furthermore, at all time points, phosphorylated AKT levels were higher in male cells than female cells (Table 1).

Interestingly, inhibiting phosphorylation of AKT with LY294002 resulted in reduction of pro-MMP-9 secretion by both male and female RASMCs at 24 hours of elastase treatment. However, treatment of cells with LY294002 did not have any effect on active MMP-2, as seen in Figure 9.

We next treated the cells with siRNA for AKT, incubated them with elastase, and performed zymography. Similar to LY294002, transfection of male RASMCs with siRNA for AKT and treatment with elastase resulted in a significant reduction in pro—MMP-9 activity. However, unlike LY294002, transfection of male RASMCs with AKT siRNA also resulted in significant decreases in pro— and active MMP-2 secretion. Again, such as LY294002, transfection of female RASMCs with AKT siRNA had no effect on secretion of pro— and active MMP-2 into the medium (data not shown).

In this series of experiments, we first used an in vivo mouse model of AAA to determine the status of AKT in male and female mice. We observed that, as the disease progressed, there was a significant increase in phosphorylated AKT in male compared with female mice. In addition, tissues from male human patients with AAA had much higher levels of phosphorylated AKT than tissues from male cadavers. Our immunofluorescence experiments showed that in both mice with AAA and human tissues from patients with AAA, p308 levels were higher than in controls and p308 costained with macrophages. Based on the in vitro experiments, we also speculate that most of the p308-producing cells were mainly the smooth muscle cells because they are the most abundant type of cells in the aorta and, to a lesser extent, by the macrophages that get recruited to the site. Likewise, we think that MMP-2 and MMP-9 are mostly secreted by SMCs, and some MMP-9 is also secreted by the macrophages at the site of AAA.

Treatment of mice with LY294002, a known AKT phosphorylation inhibitor, did not completely abolish AAA formation after elastase perfusion in mice. We speculate that LY294002 treatment did not inhibit the progression of AAA formation as the result of either the low dose of LY294002 that was used or the timing of its delivery started 4 days after AAA induction. Probably, an increased dose of LY294002 or delivery maintaining a continuous sustained release might have a greater effect on the progression of AAA in the elastase model of the disease. Also, pretreatment of mice with LY294002 for a few days before elastase perfusion may be able to arrest AAA formation. It is also possible that phosphorylation of AKT could be involved in AAA initiation but not critical for AAA progression. However, we did observe that LY294002 was able to preserve the elastin fibers in the aorta, as seen in the Verhoff’s—van Gieson staining (Figure 4). Also, LY294002 treatment resulted in less p308 and p473 staining in mouse AAA tissues (Figure 5). When we treated mouse macrophages with elastase under in vitro conditions, we observed increased levels of p308 and MMP-
9 secretion from these cells (Figure 8), which was similar to our experimental observations with vascular aortic smooth muscle cells (Figure 9 and Supplemental Figure S2). This increased production of MMP-9 from the macrophages was inhibited when the cells were treated with LY294002.

In conclusion, this study shows that, under in vitro conditions, elastase induces phosphorylation of AKT in RASMCs and there are increased levels of phosphorylated AKT in male RASMCs compared with female RASMCs. Elastase-induced differential phosphorylation of AKT in male and female RASMCs parallels differential activation of MMPs in RASMCs. Induction of MMP-9 from both male and female RASMCs by elastase is inhibited by pharmacological inhibitors of AKT phosphorylation, whereas the use of AKT siRNA resulted in inhibition of both MMP-2 and MMP-9 by male RASMCs. This study perhaps at least partially explains why AAA is more common in men than women. Although more studies are necessary, the observation that inhibition of the AKT pathway inhibits activation of MMPs may lead to the development of new drugs for the treatment of AAA.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajipath.2013.09.016.

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