Simvastatin inhibits neutrophil degranulation induced by anti-neutrophil cytoplasm auto-antibodies and N-formyl-methionine-leucine-phenylalanine (fMLP) peptide

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ABSTRACT

الأهداف: اختبار الفرضية القائلة بأن سيمفاستاتين هو قادر على منع تحبب كريات الدم البيضاء المعتدلة الناجم عن المضادات الذاتية لتلك الكريات.

الطريقة: أجريت هذه الدراسة خلال الفترة من مارس 2010م حتى سبتمبر 2011م في جامعة برمنغهام، المملكة المتحدة. تم استعمال تنقية الغلوبولين المناعي من بلازما 20 مريض تم اختيارهم عشوائياً من مرضى التهاب الأوعية الدموية الكلوية ودراسة تأثيرها على تحبب كريات الدم البيضاء المعتدلة بوجود أو عدم وجود دواء السيمفاستاتين بجرعة 10 مايكرومول. كذلك اختبرت نفس الجرعة من السيمفاستاتين لمنع مادة ف.م.ل.ب في تحبب الكريات البيضاء المحببة. بالإضافة إلى ذلك، فأن قدرة المصل تم الحصول عليها من الفئران التي تلقت السيمفاستاتين بجرعة 25 ملغم على كغم في اليوم لمنع تحبب الكريات البيضاء المحببة تم اختباره في المختبر.

النتائج: إضافة مادة السيمفاستاتين حجبت بشكل مهم قابلية الغلوبين المناعي على تحبب كريات البيضاء المعتدلة بنسبة 48% (p=0.02). حجب السيمفاستاتين كذلك بشكل مهم قابلية المادة ف.م.ل.ب بنسبة 30%, p=0.04 على تحبب كريات البيضاء المحببة. لقد أثبتنا أيضا أن المصل من الفئران التي تلقت سيمفاستاتين حجبت بشكل مهم تحبب كريات البيضاء المحببة مقارنة مع المصل من الحيوانات السيطرة (23.5%, p=0.03).

خاتمة: منعت السيمفاستاتين كلاً من الغلوبين المناعي ومادة ف.م.ل.ب على تحبب كريات الدم البيضاء المحببة. هذا يستحق أن يستخدم الستاتين في أمراض التهابات الأوعية الدموية. هذه النتائج تدعونا أن نستخدم الستاتين في علاج الأمراض الالتهابية للأوعية الدموية التي تنطوي على تحبب كريات الدم البيضاء المعتدلة للأمراض الخاصة بهم.

Objectives: To test the hypothesis that simvastatin is capable of blocking human neutrophil degranulation induced by proteinase 3 (PR3)-anti-neutrophil cytoplasm auto-antibodies (ANCA) and myeloperoxidase (MPO)-ANCA, and by the chemotactic and inflammatory peptide N-formyl-methionine-leucine-phenylalanine (fMLP).

Methods: This study was conducted between March 2010 and September 2011 at the Renal Institute of Birmingham, University of Birmingham, Birmingham, United Kingdom. Immunoglobulin G (IgG) was purified from the plasma of 20 randomly selected patients with ANCA-associated vasculitis (10 PR3- and 10 MPO-ANCA), and their ability to induce neutrophil degranulation in the presence or absence of simvastatin (10 μM) was tested. The ability of the same dose of simvastatin to block fMLP-induced neutrophil degranulation was also tested. In addition, the ability of serum obtained from rats that received simvastatin at a dose of 25 mg/kg/day to block neutrophil degranulation in vitro was tested.

Results: The addition of simvastatin significantly inhibited ANCA IgG-induced neutrophil degranulation by 48% (p=0.02). There was no significant difference in response to simvastatin inhibition (p=0.73) between PR3- and MPO-ANCA. Simvastatin also inhibited neutrophil degranulation induced by 1 μM fMLP (30%, p=0.04). We further demonstrated that serum from rats that received simvastatin significantly inhibited neutrophil degranulation induced by ANCA (31.7%, p=0.01) and fMLP (23.5%, p=0.03) compared to serum from control animals.

Conclusion: Simvastatin blocked both ANCA and fMLP-induced neutrophil degranulation. It is worth pursuing further therapeutic investigation of statins in vascular inflammatory diseases that involve neutrophil degranulation in their pathogenesis.

Saudi Med J 2013; Vol. 34 (5): 477-483

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Received 19th February 2013. Accepted 21st April 2013.

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Anti-neutrophil cytoplasmic auto-antibodies (ANCA) directed against proteinase 3 (PR3) and myeloperoxidase (MPO) are implicated in the pathogenesis of systemic small vessel vasculitis characterized by severe inflammation and necrosis of blood vessel walls affecting vital organs, such as kidney and lungs.[1-5] The binding of ANCA to MPO and PR3 on the surface of neutrophils results in an increase in neutrophil adhesion and migration through the endothelium.[6] Using mice with a humanized immune system, we recently reported a direct evidence that anti-PR3 ANCA IgG are pathogenic in vivo, and have the capacity to trigger all the hallmarks of vasculitis in mice with circulating human neutrophils.[7] In vivo animal models have also been used to confirm that ANCA directed against the neutrophil antigen MPO and neutrophils are intimately involved in development of glomerulonephritis and vasculitic lesions.[8,9] The granule enzymes, MPO, and PR3 are implicated in host defence against infectious diseases.[10,11] Bacterial infections were reported to cause small vessel vasculitis,[12,13] and play a role in the development of ANCA.[14,15] The bacterial product N-formyl-methionine-leucine-phenylalanine (fMLP) peptide is an important factor in the signal transduction of neutrophil recruitment in vascular inflammation,[16-19] and the inhibition of the fMLP receptor with either inhibitory antibodies or receptor's specific antagonist prevented neutrophils' intravascular crawling and significantly inhibited chemotaxis of neutrophil towards necrotic cells.[18,20] Human immunoglobulin (Ig)G from patients with ANCA-associated vasculitis and fMLP are potent activators of neutrophil degranulation, which is an important step in vascular inflammation.[21-23] The cholesterol lowering agents, statins, are known to have a range of anti-inflammatory effects.[24-26] Simvastatin was shown to block lipopolysaccharide-induced acute lung injury by decreasing neutrophil recruitment, and was also shown to inhibit fMLP-induced neutrophil adhesion and reactive oxygen species release.[27] Therefore, in this study we tested the hypothesis that simvastatin is capable of blocking human neutrophil degranulation induced by proteinase 3 (PR3)-ANCA and myeloperoxidase (MPO)-ANCA, and by the chemotactic and inflammatory peptide N-formyl-methionine-leucine-phenylalanine (fMLP).

Disclosure. This study was funded by the Medical Research Council (Grant # G0801025), United Kingdom.

Methods. This study was conducted between March 2010 and September 2011 at the Renal Institute of Birmingham, University of Birmingham, Birmingham, United Kingdom. All reagents and chemicals were obtained from Sigma Aldrich (Poole, UK) unless otherwise stated. Neutrophils were isolated from healthy volunteers using Percoll gradient as previously described by Toothill et al.[28] The ability of neutrophils to exclude Trypan blue was used to measure cell viability. Using the immunocytochemistry method, Percoll purified neutrophils ($1x10^5$ cells/slide) were fixed in cold ethanol and incubated with 1/20 diluted plasma from either MPO-ANCA or PR3-ANCA vasculitis patients, or with the same dilution of plasma from mesangiocapillary glomerulonephritis (MSCGN) patients as a control kidney disease. Goat anti-human IgG1 conjugated to Alexa 568 (Invitrogen, UK) and 4',6-diamidino-2-phenylindole (DAPI) to stain the nucleus were then applied, and the slides were then visualized using a Zeiss confocal LSM 510 microscope (Zeiss, Gottingen, Germany). Neutrophil degranulation was assessed by the release of myeloperoxidase as described previously by Hussain et al.[29] In brief; neutrophils at $2.5x10^6$/ml were primed with tumor necrosis factor (TNF)α and cytochalasin B to increase the expression of ANCA antigens, and incubated with 200 μg/ml IgG or 1 μM fMLP (positive control) for 15 minutes at 37^0C. Supernatants were removed and enzymatic activity was assessed by incubating them with the MPO substrate, o-phenylendiamine dihydrochloride (OPD) for 30 minutes, and assessing optical density at 450 nm. Neutrophils were pre-treated for 15 minutes with either active simvastatin (Calbiochem, Nottingham, UK), or serum from rats that received simvastatin prior to priming cells to test its inhibitory effect on degranulation assay. The corresponding controls were neutrophils pre-treated with the vehicle, or with serum from rats given normal saline. Rats were maintained in a pathogen-free barrier facility with a 12-hour light/dark cycle, and had free access to food and water. Simvastatin drug (Rosemont Pharmaceuticals, Leeds, UK) was administered daily for more than 2 weeks at a concentration of 25 mg/kg/day to Wistar Kyoto (WKY) rats (n=4 rats) by oral gavage. The same number of rats also received normal saline by oral gavage as control animals. The conversion of simvastatin drug into an active form circulating in the blood of these animals were used as a source of simvastatin to block neutrophil degranulation in response to fMLP or ANCA IgG. Human IgG samples were purified from plasma exchange effluent of patients with active anti-PR3 and

anti-MPO positive pulmonary and renal vasculitis undergoing plasmapheresis as part of routine clinical care. The plasma samples used to isolate IgGs were collected under the ethics of the South Birmingham protocol RRK2086. All fulfilled Chapel Hill consensus classification criteria for diagnosis of systemic small vessel vasculitis.[30] Control IgG was purified from plasma exchange effluent derived from a patient with MSCGN (control disease) or pooled plasma from healthy donors. Total IgG was separated from plasma using protein G affinity chromatography as previously described by Williams et al,[31] and tested in immunocytochemistry and degranulation assays mentioned above.

Statistical analysis was performed with Mann-Whitney test or the 2-tailed Student's t-test. A $p<0.05$ was considered statistically significant.

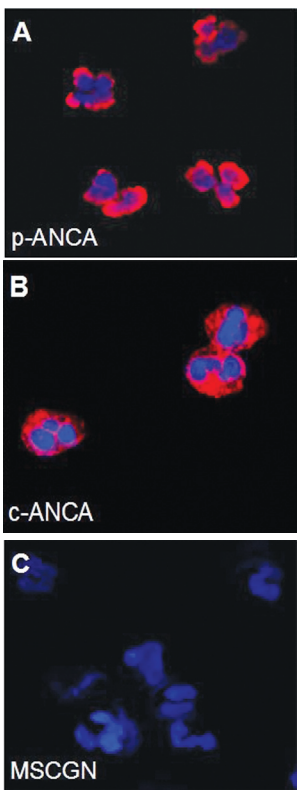

Figure 1 - Binding of anti-neutrophil cytoplasmic auto-antibodies (ANCA) to human neutrophils. Immunostaining of human neutrophils with plasma from vasculitis patients compared to plasma from the control disease, mesangiocapillary glomerulonephritis (MSCGN). The myeloperoxidase (MPO)-ANCA, proteinase 3 (PR3)-ANCA, or MSCGN diluted plasma (1/20) were added to each slide and this was followed by the addition of anti-human immunoglobulin G (red) and 4',6-diamidino-2-phenylindole to stain the nucleus blue. Perinuclear staining (**A**) and cytoplasmic staining (**B**) represent the interaction of MPO-ANCA and PR3-ANCA with human neutrophils compared to control MSCGN (**C**).

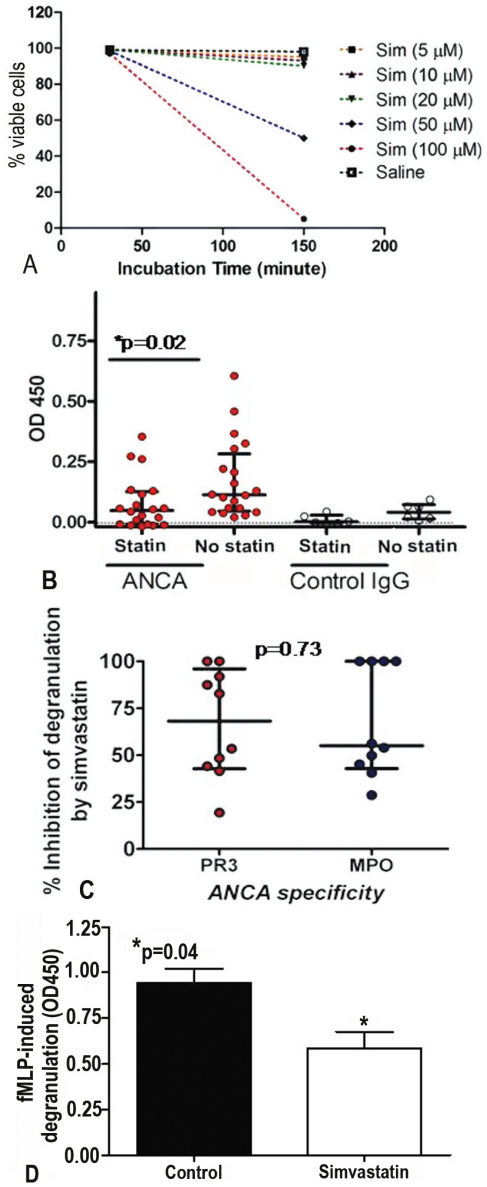

Figure 2 - Inhibition of neutrophil degranulation by active simvastatin. The effect of simvastatin on cell survival (**A**) was monitored using trypan blue exclusion assay after treating human neutrophils with different concentrations of simvastatin (5, 10, 20, 50, 100 μM) for 150 minutes. The anti-neutrophil cytoplasmic auto-antibodies (ANCA) or control immunoglobulin Gs were incubated with human neutrophils with or without 10 μM simvastatin (**B**) and the supernatants were assayed for neutrophil degranulation by the release of myeloperoxidase (MPO) (**C**). Comparison between simvastatin (10 μM) inhibitions for PR3- and MPO-ANCA-induced neutrophil degranulation. Results represent the median (±IQR), (n=20) and experiments performed in triplicate (**D**). Human neutrophils were incubated with 1 μM N-formyl-methionine-leucine-phenylalanine (fMLP) with or without simvastatin (10 μM) and the supernatants were assayed for neutrophil degranulation by the release of MPO. Results represent the mean (±SEM) (n=3) and experiments were performed in triplicate.

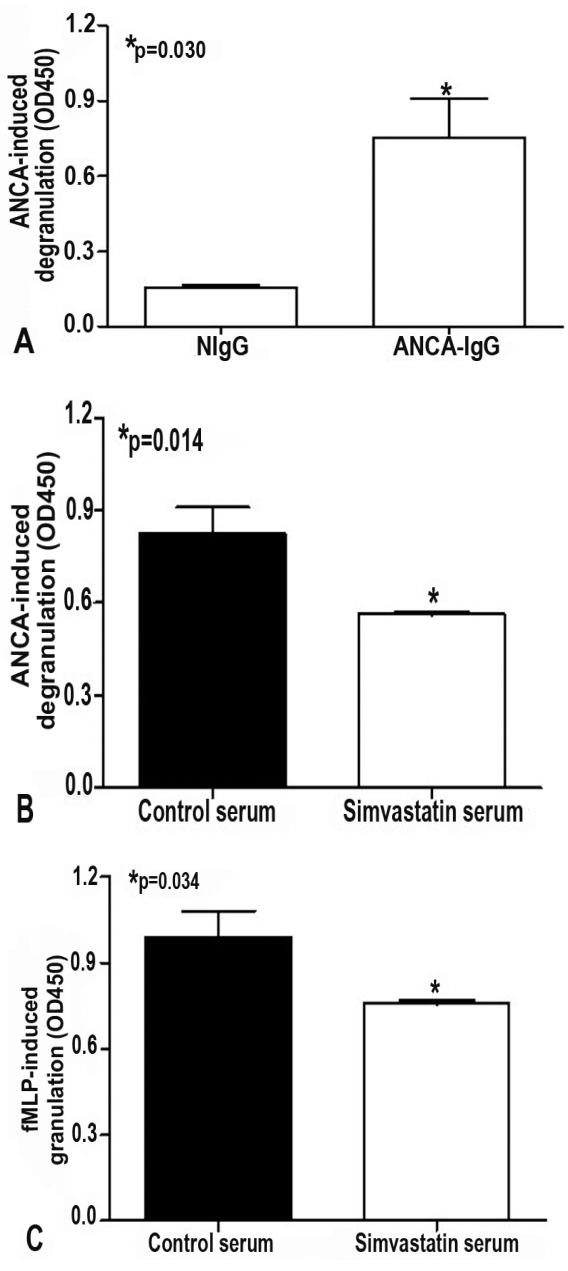

Figure 3 - Inhibition of neutrophil degranulation by serum treated with simvastatin drug. A strong neutrophil degranulator, anti-neutrophil cytoplasmic auto-antibodies (ANCA) **A**) was selected to test neutrophil inhibition by serum from animals receiving simvastatin drug. A 100 µl of neat serum obtained from rats (n=4) received simvastatin drug or control serum (n=4) was added to human neutrophils (2.5 x 10^6/ml) and incubated at 37^0C for 15 minutes before cells was primed with tumor necrosis factor-α and cytochalasin B and incubated for 15 minutes with either 200 µg/ml ANCA IgG **B**) or 1 µM N-formyl-methionine-leucine-phenylalanine (fMLP) **C**) and the supernatants were assayed for neutrophil degranulation by the release of myeloperoxidase. Results represent the mean (±standard error of mean). Experiments were performed in triplicate.

Results. In order to test the presence and capability of ANCA circulating in the blood of patients with vasculitis and binding to human neutrophils, diluted plasma obtained from patients with microscopic polyangiitis (tested positive for p-ANCA) and Wegener's glomerulonephritis (tested positive for c-ANCA) were incubated with human neutrophils in vitro. Plasma from mesangiocapillary glomerulonephritis (MSCGN) that tested negative for both p-ANCA and c-ANCA auto-antibodies was used as a disease control. Cells were then immunostained and visualized using confocal microscopy (**Figure 1**). Positive immunostaining of human neutrophils by plasma from MPO-ANCA (p-ANCA) and PR3-ANCA (c-ANCA) positive patients was observed (**Figures 1A and 1B**) but absent in the disease control (**Figure 1C**). Control neutrophils from mice failed to react with the plasma that tested positive for PR3-ANCA and weakly immunostained by plasma tested positive for MPO-ANCA (data not shown). We then purified IgGs from the plasma of 20 randomly selected patients with ANCA-associated vasculitis with high titers of auto-antibodies (10 PR3 and 10 MPO-ANCA). To determine the effect of the purified ANCA IgGs on neutrophil function and the potential inhibition of such effects by statins, we tested the ability of each IgG preparation to stimulate neutrophil degranulation in the presence and absence of simvastatin. This was assessed by measuring the release of MPO from stimulated neutrophils with a safe dose of simvastatin. The effect of increasing concentrations (5-100 µM) of simvastatin on cells viability for up to 2 hours was determined using trypan blue exclusion assay. As shown in **Figure 2A**, simvastatin of up to 20 µM was safe to use in the neutrophil degranulation assay. Compared to healthy donors IgGs, ANCA IgGs significantly (p=0.03) induced degranulation of human neutrophils that was significantly (p=0.02) inhibited with 10 µM simvastatin (**Figure 2B**). We then tested if there was a difference in simvastatin inhibition between PR3- and MPO-ANCA and found no significant response (p=0.73) (**Figure 2C**). Both fMLP and ANCA are potent inducers of vascular inflammation via neutrophil activation. Therefore, we tested the hypothesis that simvastatin may inhibit fMLP-induced neutrophil degranulation. As expected, fMLP (1µM) induced a strong neutrophil degranulation signal, and pre-incubation of cells with active simvastatin (10 µM) significantly blocked the action of fMLP by 30% (±5, p=0.04) as shown in **Figure 2D**. To further test that prescribed simvastatin drugs are converted into a functionally active form circulating in the blood

of animals that could block neutrophil degranulation induced by ANCA or fMLP comparable to the active simvastatin form (sodium derivative), serum obtained from WKY rats that received the drug were used as a source of active simvastatin compared to control serum from rats which received normal saline. Among the 20 purified ANCA IgGs, we selected the IgG (ANCA1) that gave us a strong neutrophil degranulation (**Figure 3A**) to test our hypothesis mentioned above. We found a significant inhibition of neutrophil degranulation in response to ANCA (**Figure 3B**, p=0.014) and fMLP (**Figure 3C**, p=0.034).

Discussion. This study shows that simvastatin is able to block neutrophil degranulation induced by MPO- and PR3-ANCA IgGs derived from active vasculitis patients and by the bacterial peptide, fMLP, which is also involved in neutrophil degranulation and mobilization to the vascular inflammatory site. Furthermore, this study demonstrates a comparable inhibition by simvastatin to neutrophil degranulation induced by MPO- and PR3-ANCA IgGs. Binding of auto-antibodies (ANCA) to human neutrophils shown in **Figure 1** is regarded as the first step in the pathogenesis of small vessel vasculitis causing lung injury and glomerulonephritis that leads to renal failure.[32,33] The ANCA-neutrophil complex was previously shown to slow down the movement of neutrophils inside the blood vessels and enhance neutrophil adherence followed by degranulation and release of reactive oxygen species (ROS) like superoxide that initiate endothelial and vascular injury.[6,32] We therefore tested to see if simvastatin is able to prevent neutrophil degranulation, which is involved in vascular injury. Indeed, our data shown in **Figure 2** demonstrate inhibition of neutrophil degranulation induced by ANCA with simvastatin. These results complement the previously published work on inhibition by cerivastatin and simvastatin, of superoxide generation by neutrophils stimulated with MPO- and PR3-ANCA,[34] and highlight the importance of these anti-inflammatory agents as a tool to block neutrophil degranulation and neutrophil release of ROS in vitro. The degree of inhibition by simvastatin was comparable between MPO- and PR3-ANCA induced neutrophil degranulation (**Figure 2**), whereas no conclusion can be deduced from the ROS inhibition study by statins since they only used small numbers of MPO-ANCA preparations in their study compared to PR3-ANCA preparations.[34]

Infection aggravates neutrophil degranulation and ROS release[35] and augments ANCA pathogenesis[14] through the release of proinflammatory cytokines that increase PR3 and MPO expression on the surface of neutrophils, and also through an increase in the expression of adhesion molecules on the surface of the blood vessels.[36] The PR3-ANCA increases the sensitivity of bacterial peptide, fMLP, towards neutrophils and enhances its movement,[37] and fMLP induces strong ROS release and neutrophil degranulation.[10,32] Such a relationship between ANCA and fMLP prompted us to test the hypothesis that simvastatin may inhibit fMLP-induced neutrophil degranulation, and the data in **Figure 2** and **Figure 3** clearly demonstrate a significant inhibition to this pro-inflammatory agent by simvastatin. This may have useful clinical implications. However, a recently published work[27] reported that simvastatin failed to block fMLP-induced neutrophil degranulation using FACS analysis measuring cell surface expression of DC11b, CD29, and FPRL 1. This discrepancy with our work may be attributed to their use of a higher dose of fMLP (10 μM) compared to 1 μM fMLP used in our experiments.

As shown in **Figure 2**, active simvastatin (sodium derivative) clearly inhibited ANCA- and fMLP-induced neutrophil degranulation. However, the prescribed simvastatin drug, which is only converted into an 'active' simvastatin form inside the body,[38] as expected did not block neutrophil degranulation induced by either ANCA IgG or fMLP (data not shown). The prodrug inactive simvastatin is converted by a complex process of hydrolysis and oxidation in the liver into an active form called simvastatin acid or β-hydroxy acid.[38] We therefore, tested the ability of the serum, as a source of active simvastatin, obtained from animals which received one of the commercially available simvastatin drugs (see methods section) at a concentration of 25 mg/kg/day to inhibit neutrophil degranulation. Using high simvastatin dose was justified to compensate for the very low bioavailability of the drug (5%)[38] to make it relatively comparable to the 10 μM simvastatin that we used here and in previous study by Choi et al[34] to block ANCA-induced superoxide release by human neutrophils. Indeed, pre-treatment of human neutrophils with serum from rats, which received simvastatin (**Figure 3**) clearly inhibited degranulation in vitro induced by fMLP (23.5%) and ANCA (31.7%). These findings are comparable to a previous study that used serum obtained from hypercholesterolemic patients on simvastatin as a source of active simvastatin, which inhibited (24.5%) human smooth muscle cell proliferation in vitro.[39] Furthermore, the degree of fMLP inhibition by serum from rats (23.5%) was comparable to active simvastatin (26%), whereas a

higher inhibition of ANCA1-induced degranulation was obtained by active simvastatin (39%) compared to (31.7%) inhibition obtained with serum from animals treated with simvastatin drug (**Figure 2** and **Figure 3**).

We believe that further future studies need to be carried out to investigate the potential signalling pathways involved in the mechanism of simvastatin inhibition such as PKC, Rho, p38MAPK, and whether the inhibition process is through reducing neutrophil membrane expression of ANCA antigens. In conclusion, the findings in this study could pave the way to the use of simvastatin in animal models to study vascular inflammatory diseases that involve neutrophil degranulation in their pathogenesis.

Acknowledgment. *The author gratefully acknowledges and highly appreciates the support and input of Prof. Mark Little and the technical help of Dr. Hamad Al-Nuaimi from the Renal Institute of Birmingham, College of Medicine and Dentistry, University of Birmingham, Birmingham, United Kingdom.*

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Illustrations, Figures, Photographs

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