Assessment of Endothelial Function Using Peripheral Waveform Analysis
A Clinical Application

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OBJECTIVES The study was done to determine whether radial artery applanation tonometry can be used as a noninvasive method of assessing global endothelial function.

BACKGROUND It is known that β₂-receptor stimulation results in endothelial release of nitric oxide. Furthermore, for over a century glyceryl trinitrate (GTN) has been shown to markedly affect the arterial pressure waveform, even in the absence of significant blood pressure (BP) changes. Therefore, it was hypothesized that the change in the peripheral pressure waveform, as measured using tonometry and quantified using the augmentation index (AIx) and in response to Salbutamol (Salb), would allow assessment of global endothelial function.

METHODS The study contained three parts. In the first study, Salb (400 µg) was administered to 11 healthy subjects via inhalation after either intravenous N-ω-nitro-monomethyl-L-arginine (L-NMMA) (3 mg/kg over 5 min) or control solution (normal saline) in the supine, rested, fasted condition. The BP, heart rate and waveform responses were recorded each 5 min following Salb for 20 min. Next, GTN was given and responses recorded 5 min later. In the second study, both the reproducibility of Salb and the GTN responses were assessed in 9 subjects studied twice on separate days. In the third study, the Salb and GTN responses of 12 subjects with angiographic coronary artery disease (CAD) were compared with 10 age-matched control subjects with no atherosclerotic risk factors.

RESULTS After control infusion, AIx decreased following Salb, from 50.8 ± 4.3% to 44.8 ± 4.2%, a change of −11.8 ± 3.7%, p < 0.01. After L-NMMA, AIx did not significantly change following Salb (54.2 ± 5.1% vs. 52.9 ± 5.3%, −2.0 ± 3.1%). The GTN-induced decreases in AIx were similar after either infusion (35.1 ± 3.3% vs. 36.5 ± 3.3%). Reproducibility of Salb-induced changes in AIx between studies performed on separate days was good (r = 0.80, p < 0.01). Salb-induced changes in AIx in CAD patients were significantly less compared to control subjects (−2.4 ± 1.9% vs. −13.2 ± 2.4%, respectively, p < 0.002). The GTN-induced changes were not significantly different (−27.6 ± 4.2 vs. −38.9 ± 4.4%, p = 0.07).

CONCLUSIONS The peripheral arterial pressure waveform is sensitive to β₂-stimulation. Changes are related to nitric oxide release, are reproducible and can distinguish between clinical subject groups. Arterial waveform changes following Salb may thus provide a noninvasive method of measuring "global" arterial endothelial function. (J Am Coll Cardiol 2002;40:521–8) © 2002 by the American College of Cardiology Foundation

Nitric oxide (NO) is recognized to be a potent smooth muscle relaxant responsible, in large part, for vasodilation in response to stimulation of an intact endothelium (1). The obligatory role of the endothelium in vasodilation offers a mechanism whereby arterial "health" can be assessed prior to the development of atheroma. Studies have confirmed that endothelial dysfunction occurs prior to atheroma formation (2), and assessment of endothelial function has been used as a surrogate marker for arterial damage (3–5). Although these studies have examined endothelial function in the brachial artery (by ultrasound measurement of brachial artery flow-mediated dilation in response to reactive hyperemia), fundamental to the significance of the technique is the close relationship demonstrated between peripheral endothelial dysfunction and coronary atheroma (6,7).

Applanation tonometry is a method whereby the arterial pressure waveform of the artery under study can be obtained noninvasively. The technique is based on that pioneered for ocular tonometry for measurement of intraocular pressure by flattening the globe of the eye (8). The technique involves positioning the tonometer (a small pencil probe-like device) over the maximal arterial pulsation to minimally flatten (aplanate) the arterial wall. By normalizing the circumferential stresses, the electrical resistance of a small piezoelectric crystal within the tip of the tonometer varies directly with intra-arterial pressure, allowing accurate recording of the pressure waveform. The accuracy of the waveform registration has been validated in animals and humans (9,10). It has been extensively used in human population studies, and is widely applicable because it is noninvasive (10–14).

It has recently been shown that β₂-receptor stimulation results in endothelial release of NO both in animals (15,16) and humans (17). Furthermore, for over a century

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exogenous NO, in the form of glyceryl trinitrate (GTN), has been known to markedly affect the peripheral pressure waveform even in the absence of significant brachial blood pressure (BP) changes (18). It was hypothesized that systemic Salbutamol (Salb) might induce widespread endothelial release of NO, thus changing the peripheral pressure waveform and therefore allowing assessment of the global endothelial function.

METHODS

The study, approved by the Research Ethics Committee of the Royal Brompton and Harefield National Health Service Trust, consisted of three parts. Study 1 investigated the effect of NO synthase inhibition on Salb- and GTN-induced changes in the augmentation index (AIx) as measured by radial applanation tonometry. Study 2 assessed the reproducibility of Salb and GTN responses, and study 3 compared the Salb and GTN AIx responses of subjects with and without angiographically proven coronary artery disease (CAD).

Study protocols. STUDY 1: PROTOCOL VALIDATION. Eleven healthy male subjects aged 23 to 36 years (29.1 ± 1.4 years, mean ± SEM) without known cardiac disease, history or presence of hypertension on examination, hypercholesterolemia or regular medications were enrolled. Mean cholesterol was 4.4 ± 0.3 mmol/l; mean body mass index was 23.8 ± 1.0 kg/m². One subject had a history of occasional smoking. Subjects were studied twice on separate days, 6 h fasting, and supine. On both days, subjects had an intravenous cannula (20G) placed in the contralateral hand to the study artery. After cannulation, a normal saline infusion was commenced at 2 ml/min. After 15 min of saline infusion, subjects were randomized to receive either Nω-nitro-monomethyl-L-arginine (L-NMMA) (3 mg/kg over 5 min) or additional saline for a further 5 min. Once the infusion was completed, subjects were given Salb 400 µg by supervised inhalation (Ventolin Rotacap, Allen & Hanbury’s, Uxbridge, United Kingdom) after initial demonstration. The empty cap was inspected after each inhalation to ensure complete drug delivery. At study completion, after 20 min, sublingual GTN (250 µg) was given. The operator was blinded to the infusion regimen. The study was repeated with the alternate infusion protocol on a second study day.

STUDY 2: REPRODUCIBILITY. Nine healthy male subjects aged 35.4 ± 3.4 years were studied twice on separate days. Baseline measurements were taken for 15 min (four recordings). Subjects were then given Salb 400 µg inhalation in an identical fashion to study 1. Recordings were taken for an additional 20 min, and then sublingual GTN 250 µg was given and the measurements taken for a final 5 min.

STUDY 3: CLINICAL VALIDATION. Salbutamol-induced pressure waveform responses in 12 male patients with angiographically proven CAD (aged 49.0 ± 1.3 years) were compared with 10 age- and gender-matched healthy volunteers with no known risk factors for atherosclerosis. All vasoactive medications were stopped for at least 24 h in the CAD group. Subjects in the healthy control cohort were not taking regular vasoactive medications. Subjects were studied with an identical protocol to study 2. One control subject was found to have fasting hypercholesterolemia (total cholesterol 6.2 mmol/l) and was therefore excluded from further analysis. Results are presented for the remaining 10 control subjects with no atherosclerotic risk factors.

MEASUREMENTS. All subjects were studied supine after a minimum of 6 h of fasting. The BP was measured noninvasively every 5 min throughout the study period using Dynamap recordings on the contralateral arm (Critikon, Tampa, Florida). Immediately after each BP measurement, radial artery pulse recordings were taken to determine heart rate (HR) and AIx as previously described (11). The radial arterial pressure waveform was recorded directly onto a laptop computer (Toshiba Satellite 2520CDT), running proprietary waveform analysis software (SphygmoCor Px Version 6.0, ATCOR, Sydney, Australia). This automatically averages approximately 10 waveforms and calculates AIx on the averaged waveform. Drug infusions were rate-controlled using an infusion pump (IVAC, Alaris Medical Systems, San Diego, California). Baseline BP, HR and AIx were taken as the mean of the final two recordings prior to either L-NMMA or Salb administration.

The end point used to assess endothelial responses was the “augmentation index,” a term initially used by Kelly et al. (11). This represents a quantification of the reflected arterial wave to the primary arterial wave. It is calculated as the ratio of the pulse pressure at the second systolic peak to that at the first systolic peak. Although it has been used to assess central and peripheral pressure waveforms previously, the peripheral responses were specifically examined in this study. The major reason for using the peripheral waveform is that it is less susceptible to filtering, and the secondary wave is more easily detected by the automated software. There were no subjects in this group in whom a secondary waveform was not detected for baseline or Salb. Because GTN so effectively abolishes the reflected arterial wave, the dicrotic notch was used as the height of the second wave in
some patients. Previous studies have confirmed that the AIx is dependent on arterial function (19). It has been shown to change markedly with aging, attributed to increased wave reflection (11), as well as being very sensitive to the vasodilatory effects of GTN (20). This study assessed whether endothelial stimulation by Salb had a similar effect on the pressure waveform due to beta2-receptor-stimulated endothelial NO release.

DRUGS. In this study, beta2-adrenoceptor stimulation by Salb was used to assess endothelial responses. Previous studies have shown that Salb induces potent release of NO (approximately 50% increase in local flow, similar in magnitude to the effect of acetylcholine) in response to intra-arterial infusion (15,17). It has recently been shown that inhaled Salb may also induce endothelium-dependent vasodilation (21). The role of the endothelium was confirmed in those studies by showing antagonism by L-NMMA, as in the current proposal. The dose of 0.6 mg/kg/min L-NMMA represents a middle-range dose from earlier studies (22). Previously, L-NMMA has been shown to achieve maximum effect within 10 min, providing peak NO synthase antagonism at a similar time to expected peak Salb effect. All subjects were given GTN at the end of the study, as this has the longest half-life, and vascular response has previously been shown to be independent of endothelial function (2).

STATISTICS. In study 1, measurements following Salb and GTN were compared to their respective baseline. To determine whether there was an effect of L-NMMA on the results, the percentage change in each parameter following saline was compared to the percentage change following L-NMMA and analyzed using two-way analysis of variance (ANOVA) with replication. In study 2, results for visit 1 and visit 2 were examined using correlation analysis and displayed according to Bland-Altman plots (23). In study 3, continuous variables were assessed using unpaired t tests and dichotomous variables using chi-square tests. Data are presented as mean ± SEM. A p ≤ 0.05 was considered significant.

RESULTS

Study 1: protocol validation. Salbutamol inhalation was associated with significant changes in the radial pressure waveform without significant change in mean BP; GTN was associated with even more marked waveform changes, and was also associated with a slight decrease in mean BP. An example of Salb and GTN waveform changes in a single individual is shown in Figure 1. Over the entire group, Salb administration was associated with an 11.8 ± 3.7% decrease in AIx when given after the control saline solution (Fig. 2A). When Salb was given after L-NMMA, AIx did not change (−2.0 ± 3.1%). There was no significant interaction effect between the saline and L-NMMA infusions and the change following Salb or GTN administration (p = 0.10, for two-way ANOVA). Decreases in AIx following GTN were significantly greater than that following Salb and were similar following either saline or L-NMMA (−35.1 ± 3.3% and −36.5 ± 3.3%, respectively; Fig. 2B). There was no significant effect of either Salb alone or L-NMMA + Salb on mean BP (Fig. 2C, p = 0.06). Glyceryl trinitrate was associated with a slight decrease in BP in both cases (Fig. 2D). Salbutamol was associated with a significant increase in HR, which was blocked after L-NMMA infusion (Fig. 2E). It appears likely that the blocked HR response was a
direct effect, as BP did not change significantly following L-NMMA infusion in this study.

Whereas Salb inhalation was associated with both a decrease in AIx and an increase in HR over the entire group, no correlation was seen between these two physiological parameters in the same patients ($r = 0.11$, $p = 0.75$; Fig. 3). In view of the known relationship between HR and AIx in population studies (12), the lack of association between the AIx and HR in this study suggests that the changes seen in response to Salb are not simply related to HR changes alone.

**Study 2: reproducibility.** Good reproducibility occurred between baseline AIx and mean BP recordings performed on separate days (respective correlation coefficients $0.79$ and $0.73$, both $p < 0.05$). Salbutamol-induced changes in AIx were also reproducible ($r = 0.80$, $p < 0.01$). The mean change due to Salb for the two days was $16.2 \pm 3.3\%$ and $17.1 \pm 3.2\%$, and following GTN $34.5 \pm 6.0\%$ and $34.8 \pm 3.7\%$. As shown by the Bland-Altman analysis (Fig. 4) the mean difference between visit recordings was $0.9 \pm 2.0\%$.
There was no systematic error according to magnitude of Salb-induced change in AIx. The reproducibility of GTN-induced changes in AIx was also good ($r = 0.81$, $p < 0.01$). There was no effect of Salb on BP in either group, and no differences in response to Salb between the groups. A significantly greater increase in HR occurred following Salb in the control group compared to the CAD group ($8.2 \pm 1.8\%$ vs. $2.6 \pm 1.7\%$, $p = 0.04$). Despite this, no correlation existed between the change in HR in either the controls or the CAD patients and the change in AIx following Salb. There was a trend to a greater GTN response in the control group, although this was not significant ($38.9 \pm 4.4\%$ decrease vs. CAD $27.4 \pm 4.2\%$ decrease, $p = 0.07$; Fig. 5).

**DISCUSSION**

This study shows that the peripheral arterial pressure waveform is sensitive to beta2-stimulation as delivered noninvasively by inhalation. The changes induced are related to NO release, are reproducible, and can distinguish between clinical subject groups. As such, the analysis of Salb-induced changes in the peripheral arterial pressure waveform may provide a noninvasive method of assessing “global” arterial endothelial function. The potential benefits of this technique are that it is noninvasive, readily portable and is quickly learned. It thus provides a method for assessing endothelial function that may be suitable for large-scale population studies. A further benefit of this technique is that the use of Salb to induce endothelial NO release will allow a global assessment of endothelial function rather than assessment of a single vascular bed (usually the brachial arterial tree). This has not previously been available;

**Study 3: clinical validation.** The demographics for the two groups in the clinical validation study are shown in Table 1. Subjects were well matched for age with similar resting HRs and systolic BPs. Subjects with CAD had higher baseline AIx, diastolic BP rates, and had greater BMI. Salbutamol administration was associated with a significantly greater decrease in AIx in the control group compared to subjects with CAD ($13.2 \pm 2.3\%$ compared to $2.4 \pm 1.9\%$ decrease, $p < 0.002$; Fig. 5). There was no effect of Salb on BP in either group, and no differences in response to Salb between the groups. A significantly greater increase in HR occurred following Salb in the control group compared to the CAD group ($8.2 \pm 1.8\%$ vs. $2.6 \pm 1.7\%$, $p = 0.04$). Despite this, no correlation existed between the change in HR in either the controls or the CAD patients and the change in AIx following Salb. There was a trend to a greater GTN response in the control group, although this was not significant ($38.9 \pm 4.4\%$ decrease vs. CAD $27.4 \pm 4.2\%$ decrease, $p = 0.07$; Fig. 5).

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Values are mean ± SEM.

AIx = augmentation index; BMI = body mass index; BP = blood pressure; HR = heart rate; TVD = triple-vessel coronary artery disease.
controls:

the arterial tree and the left ventricle (36). An important central waveform is determined by the interaction between the shape of the central (aortic) pressure waveform. This waveform is determined not only by local factors, but also by Radial tonometry overview.

atherosclerosis (35). An important response is similar to that previously described in studies increasing in AIx in response to Salb. Such a paradoxical response is similar to that previously described in studies increasing in AIx in response to Salb (35). In three patients with CAD, there was a small GTN studies (again, one-third, 6.0% vs. 17.5%, respectively) (35). Despite the success of the currently available techniques, we sought to develop a new protocol for the assessment of endothelial function. The rationale for doing so is that the tonometry equipment is relatively simple, the technique of tonometry recording is quickly learned and the analysis is operator independent. The problems with the current techniques are, first, that the photoplethysmographic technique requires arterial cannulation for infusion of vasoactive mediators and is therefore invasive (32). This limits subject acceptability, which is particularly a problem in long-term follow-up studies.

The second major alternative, brachial ultrasound, is limited by the expense of the machine, the long learning period to obtain reliable results (33), and the axial limit of resolution of the ultrasound scanner, which is close to the change in diameter expected in response to flow-mediated dilation (34). Although the relative magnitude of waveform change due to Salb is small compared to GTN (approximately one-third, 13.2% vs. 38.9% in the clinical validation control group), it is actually very similar to the difference in magnitude of change seen in flow-mediated dilation and GTN studies (again, one-third, 6.0% vs. 17.5%, respectively) (35). In three patients with CAD, there was a small increase in AIx in response to Salb. Such a paradoxical response is similar to that previously described in studies using flow-mediated dilation in subjects with risk factors for atherosclerosis (35).

Radial tonometry overview. The radial artery pressure waveform is determined not only by local factors, but also by the shape of the central (aortic) pressure waveform. This central waveform is determined by the interaction between the arterial tree and the left ventricle (36). An important phenomenon in the genesis of the central pressure waveform in humans is reflection of the ejected pulse back from the periphery within the duration of cardiac ejection (37). This changes the shape of the central waveform as it is transmitted to the periphery. It has been shown that agents such as GTN decrease wave reflection almost completely and are associated with significant changes in the central pressure pulse (20). These changes in the central pulse are then transmitted to the radial pulse and can be measured using radial artery tonometry. The consistency of the transmission characteristics of the brachial arterial tree under the influence of GTN has been confirmed (38). Although it is possible to generate a central pressure pulse from that measured at the periphery (38,39), the changes in the central pulse waveform in response to Salb are more subtle than those seen at the periphery.

It has recently been shown that the digital volume pulse can be recorded using photoplethysmography (21). In that study, similar Salb-induced responses were shown for the digital pulse, although baseline and Salb-induced reproducibility results were not available. The present study extends these findings to demonstrate that differences in peripheral pulse responses may be seen in patients with angiographically proven CAD. Whereas the digital volume pulse is similar in shape to the pressure waveform recorded using radial tonometry, we believe the tonometrically recorded pulse can be reliably recorded and is less susceptible to temperature change than the digital volume pulse. A recent study comparing the two techniques overcame this by studying all subjects in a warmed, controlled environment (26°C) (40).

Clinical validation study. The difference seen in the Salb response in the CAD patients compared to the age-matched controls suggests that this technique may be useful in clinical trials. This study did not compare other methods for measurement of endothelial function such as flow-mediated brachial artery dilation with tonometry, although this could easily be performed in future studies. Although systolic BP was similar between the two groups, diastolic pressure was

Figure 5. Comparison of Salbutamol and glyceryl trinitrate (GTN)-induced changes in augmentation index (AIx) in coronary artery disease patients (CAD) with control subjects. A significantly greater decrease occurred in AIx in control subjects compared to CAD patients following Salbutamol (−2.4 ± 1.9%; controls: −13.2 ± 2.3%, p < 0.002). This difference was not significant following GTN (−27.4 ± 4.2 vs. −38.9 ± 4.4%, p = 0.07).
higher in the patient group. This is not surprising, as all vasoactive medications had been withdrawn, and six of the patients were currently being treated for hypertension. The greater body mass index (BMI) is also in keeping with obesity as a risk factor for CAD (41). No correlation existed between weight or BMI and Salb-induced AIx response. The lower resting AIx and HR in the control subjects are consistent with a greater degree of cardiovascular fitness. Previous studies have shown that these factors are associated with changes in aerobic capacity (13). It is possible that the lower GTN response in the CAD patients reflected impaired smooth muscle dilation. Over the entire clinical validation cohort (n = 22), a significant relationship existed between the Salb response and the GTN response (r = 0.62, p < 0.005). Within either the controls or the CAD patient groups in isolation, no such relationship was evident. Such a relationship has been previously demonstrated in a meta-analysis of studies of 800 subjects from a single laboratory using flow-mediated dilation (35).

**Study limitations.** In these pilot studies, the study groups were small in number. This, and the smaller effect of Salb compared to GTN, resulted in the nonsignificant two-way ANOVA results. To avoid complicating effects of endogenous hormones, only males were studied. Therefore, this work needs to be extended to examine the effects of Salb on the AIx in women. Although the reproducibility of this technique was very good in the current study, reproducibility may need to be further assessed in an older population, one more representative of target study populations. Further trials in various disease and risk-factor categories (hypercholesterolemia, diabetes, hypertension, smoking) are needed to determine whether the technique will be sensitive to isolated risk-factor analysis. In view of the recently recognized effect of beta2-receptor polymorphisms on resting and exercise BP responses to beta2-agonist infusions (42,43), additional studies are required to examine the effect of common polymorphisms on the inhaled Salb AIx response, both in healthy and in diseased populations.

**Conclusions.** In this study we have shown that the peripheral arterial pressure waveform is sensitive to beta2-receptor stimulation by low-dose inhaled Salb. These changes appear to be related to NO release; the technique is sufficiently sensitive to detect differences between small clinical subject groups and are reproducible over the short term. Arterial waveform changes following Salb may provide a noninvasive method of measuring “global” arterial endothelial function, and further studies are needed to document effects in larger patient groups and to examine the effect of therapies. The portability and simplicity of the technique make large-scale population studies possible.

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