Aprotinin reduces nitric oxide production in vitro and in vivo in a dose-dependent manner

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1. Cardiopulmonary bypass is associated with an increase in nitric oxide concentrations, and plasma levels of tumour necrosis factor and interleukin-1. Aprotinin, a serine protease inhibitor, commonly used during cardiopulmonary bypass to reduce blood loss, has been demonstrated to exhibit significant anti-inflammatory effects during and after cardiopulmonary bypass.

2. Airway nitric oxide was measured during cardiopulmonary bypass in 10 controls (Group 1), 10 subjects receiving half-dose aprotinin (Group 2) and 10 patients receiving full-dose aprotinin (Group 3). In vitro, a murine bronchial epithelial cell line (LA-4) was cultured with cytomix (a combination of tumour necrosis factor, interleukin-1, and (γ) interferon) with and without aprotinin in increasing concentrations. Nitrite concentrations, the stable and measureable end-product of nitric oxide oxidative metabolism, were measured in the culture supernatant by chemiluminescence.

3. Airway nitric oxide concentrations were increased after 50 min cardiopulmonary bypass compared with that measured at 5 min in controls (53 ± 5 versus 29 ± 3 ppb, P < 0.05) but not in the aprotinin-treated groups (25 ± 4 versus 14 ± 5, Group 2; 21 ± 6 versus 15 ± 3 ppb, Group 3).

4. In a dose-dependent manner, nitrite levels (means ± S.E.M.) were significantly reduced by aprotinin at 500 and 1000 units/ml when compared with cells cultured in the presence of cytomix alone (P < 0.05).

5. These data demonstrate that aprotinin, in a dose-responsive manner, reduces nitric oxide production in vitro and reduces cytokine-induced nitrite production by murine bronchial epithelial cells in vitro. Since increased airway nitric oxide is found in inflammatory lung diseases, like asthma, and anti-inflammatory therapy reduces the concentration of airway nitric oxide, these data support the concept that aprotinin is anti-inflammatory during cardiopulmonary bypass.

INTRODUCTION

Airway nitric oxide (NO) concentration, probably derived from bronchial epithelium [1,2], is increased during lung inflammatory states, like asthma [3], and cardiopulmonary bypass (CPB) [4] which target the bronchial epithelium. Elevated airway NO concentrations during asthma exacerbation [3] and CPB [4] are decreased by anti-inflammatory (glucocorticoid) therapy. Both asthma [5] and CPB [4] are also characterized by increases in measurable levels of the cytokine, tumour necrosis factor-α (TNF-α). Since airway NO concentrations may serve as a marker for lung inflammatory states characterized by bronchial epithelial inflammation [3], airway NO was measured during CPB to evaluate CPB-induced bronchial epithelial inflammation. A comparison group of patients was given aprotinin before and during CPB because of previous reports that aprotinin [6], like glucocorticoids [4], reduces systemic cytokine levels when used in conjunction with CPB in man. Since cytokines like TNF-α induce expression of inducible nitric oxide synthase (iNOS) and thus increase endogenous NO production [7], the effects of aprotinin on endogenous lung (airway) NO production were evaluated.

In addition, to further evaluate the mechanism of the in vivo effects of aprotinin on airway NO production, the in vitro effects of aprotinin on cytokine-induced nitrite production (the stable and measureable end-product of NO metabolism) by a murine bronchial epithelial cell line were studied.

Recent studies demonstrate increasing NO generation with CPB duration [4,8] and increased lung tissue expression of iNOS after CPB [9]. In addition, NO-induced lung and bowel vascular injury correlate with tissue iNOS expression [10]; thus NO tissue cytotoxicity is thought to be secondary to elevated endogenous concentrations of NO derived from iNOS activation [10]. In addition, elevated endogenous NO levels have been implicated in myocardial reperfusion injury [11]. Since aprotinin reduces myocardial injury after CPB [12], reduction of cytokine-induced iNOS expression and subsequent reduction of endogenous NO generation may prove to be a mechanism of reduced organ injury after CPB by aprotinin therapy [12].

METHODS

After obtaining Institutional Review Board approval and patient informed consent, 30 male patients scheduled for elective aortocoronary bypass surgery...
were randomized equally according to a computer generated sequence to one of three groups: (1) a control group (Group 1); (2) a group receiving half-dose aprotinin, 140 mg (1 x 10^6 KIU) intravenously as a loading dose, 140 mg (1 x 10^6 KIU) ‘pump prime’ and 35 mg (2.5 x 10^5 KIU) h constant infusion until chest closure (Group 2); and (3) a group receiving full-dose aprotinin, 280 mg (2 x 10^6 KIU) intravenously as a loading dose, 280 mg (2 x 10^6 KIU) ‘pump prime’ and 70 mg (5 x 10^5 KIU) h constant infusion until chest closure (Group 3). A pharmacist was made aware of the group assignment and supplied the anaesthesia team with either a saline placebo (Group 1) or aprotinin (Groups 2 and 3). Patients with a previous history of asthma, smoking or steroid exposure within the previous 6 months were excluded. No patient received intravenous nitroglycerine before or during the study periods. All patients in both groups were receiving chronic oral nitrate therapy, while several in both groups were also receiving chronic angiotensin-converting enzyme inhibitor therapy.

On the morning of surgery, each patient was given morphine sulphate (0.1 mg/kg) and scopolamine (0.2–0.4 mg) intramuscularly before admission to the operating room. On arrival, a radial artery catheter, a right internal jugular vein pulmonary artery catheter and large-bore intravenous lines were placed. Standard anaesthesia consisting of fentanyl (50–100 µg/kg) as a bolus and fentanyl (0.5–1.0 µg/kg) per min, midazolam (0.5–1.0 mg/kg), and acepromazine (0.05–0.1 mg/kg) was used. CPB was completed with a centrifugal pump (Biomedicus, Inc., Eden Prairie, MN, U.S.A.), hollow-fibre membrane oxygenator (Baxter Health Care, Irvine, CA, U.S.A.) with arterial line filtration and mild hypothermia (32 °C core temperature). Perfusion flow rate and mean arterial pressure during CPB were maintained between 2.2 and 2.4 l·min^-1·m^-2, and 60–80 mmHg respectively. Myocardial preservation was achieved through both antegrade and retrograde administration of cold hyperkalaemic blood (8:1, blood to crystalloid mixture) cardioplegia. Anticoagulation was obtained by the return of the activated clotting times to baseline units of total heparin administration, and confirmed by the return of the activated clotting times to baseline values.

Airway NO was measured by placing a Teflon-coated catheter inside the endotracheal tube to, but not beyond, the distal tip of the endotracheal tube. Airway gas was sampled over a 60-s time period at four intervals: (1) 5 min after institution of CPB, (2) 20 min after CPB, (3) 35 min after CPB, and (4) 50 min after CPB. Airway gas was also sampled in each subject 15 min before CPB institution after 12–15 s of apnoea. Upon institution of CPB, the endotracheal tube remained connected to the anaesthesia circuit, mechanical ventilation was discontinued and the lungs were left unventilated with zero end-expiratory pressure, and no fresh gas or oxygen flow. Airway NO concentrations were measured with a chemiluminescence analysis technique (Nitric oxide analyser model 270 NOA; Sievers Instruments, Inc., Boulder, CO, U.S.A.). The sample flow rate of the analyser was 500 ml/min. On-line recording of airway NO concentration was made on a chart recorder calibrated with a NO standard (Scott Specialty Gases, Inc., Plumsteadville, PA, U.S.A.) of 113 ppb. The amount of NO produced was calculated from the chart recording by the area under the curve in each 60-s period and is expressed as mean NO (ppb) concentration.

Cell cultures

The murine lung epithelial cell line, LA-4, was purchased from American Type Culture Collection (Rockville, MD, U.S.A.). The LA-4 cells were cultured in 6-well tissue culture plates (Costar, Cambridge, MA, U.S.A.) with Ham’s F-12 with 15% fetal calf serum until confluent. After washing three times, the LA-4 cells were cultured in serum-free Dulbecco’s modified Eagle’s medium. The cells were induced to produce NO by stimulation with cytokina, a combination of human TNF-α (10 ng/ml; Sigma, St. Louis, MO, U.S.A.), human interleukin-1β (10 ng/ml; Sigma), and murine γ-interferon (10 ng/ml; Sigma). Aprotinin (Traysol®*, Bayer, Inc., West Haven, CT, U.S.A.) was added 30 min before stimulation. Viability was assessed by Trypan Blue exclusion and was always >95%.

Nitrite determinations

Nitrite was determined by converting nitrite to NO under acidic conditions as previously described [13]. NO was measured under a nitrogen stream using a chemiluminescence analyser (Sievers model 270B, Sievers, Boulder, CO, U.S.A.). The area under the curve was measured and nitrite determined by comparison to a standard curve.

Statistics

All results are reported as means ± S.E.M. A repeated-measures analysis of variance was carried out to distinguish within-group differences over time, and t-tests were performed to evaluate differences at the same time periods between groups: P values of 0.05 or less were considered significant.

RESULTS

There were no significant differences between Groups 1, 2 or 3 in age (66 ± 3, 61 ± 4.5 and 62 ± 1.8 years respectively), weight (76.3 ± 3, 80.5 ± 3.6 and 80.1 ± 4.0 kg respectively) or CPB duration (93 ± 3.1, 99.4 ± 5 and 95.5 ± 4.5 min respectively) (mean ± S.D.).
Airway NO concentrations were increased after 50 min CPB compared with that measured at 5 min in controls (53 ± 5 versus 29 ± 3 ppb, *P < 0.05) but not in the aprotinin-treated groups (25 ± 4 versus 14 ± 5 ppb, Group 2; 21 ± 6 versus 15 ± 3 ppb, Group 3) (Figure 1). Airway NO after 15 s of apnoea pre-CPB was usually not measurable and therefore is not displayed.

**Nitrite levels**

Nitrite concentrations in the culture supernatant fluids of the LA-4 cells were elevated in the presence of cytomix. In a dose–response manner, nitrite levels (means ± S.E.M.) were significantly reduced by aprotinin at 500 and 1000 units/ml in cytomix-stimulated cells when compared with cells cultured in the presence of cytomix alone (*P < 0.05) (Figure 2).

**DISCUSSION**

Kobzik et al. [1] demonstrated by immunostaining that iNOS, but not constitutive nitric oxide synthase (cNOS), is present in human bronchial epithelium. Cytokines, including TNF-α and interleukin-1, induce increased iNOS expression in bronchial epithelial cells [2]. Inducible NOS is not normally expressed in most tissues, but requires induction by endotoxin or pro-inflammatory cytokines [14]. While some investigators report that cytokine-induced iNOS expression requires up to 3 h [15], others report a much shorter time period (2–3 min [16], up to 2 h [17]). Our data demonstrate rapidly increasing airway NO levels measured in vivo during CPB and are consistent with other reports [16] of a short time duration required for cytokine-induced iNOS activation. Others [9] have found significant increases in lung tissue iNOS expression immediately after CPB. In addition, Ruvolo et al. [8] found significant elevation of plasma nitrite concentration, when compared with baseline, as early as 45 min after the onset of CPB. These reports [8,9,16,17] demonstrate, as in this study, that cytokine-induced iNOS expression may occur over a shorter time than originally thought. Consistent with our data, Stitt et al. [18] have demonstrated that airway NO derives from iNOS, that airway NO increases immediately after reduction of pulmonary blood flow (as occurs during CPB), and that airway NO is a biomarker of endotoxin-induced lung injury.

Induction of iNOS results in much larger (nanomolar) amounts of endogenous NO compared with the smaller or picomolar levels resulting from the action of cNOS [7]. Elevated endogenous NO levels may result in airway oedema [19] and lung injury [20]. Elevated airway levels of NO have been found during lung inflammatory conditions, like asthma, that target the bronchial epithelium [3]. Recent evidence suggests that the lung inflammation during CPB similarly involves the bronchial epithelium as demonstrated by increasing concentrations of airway NO with increasing CPB duration [4]. Glucocorticoids inhibit TNF-α-induced iNOS expression in bronchial epithelium [2] and reduce airway NO levels during asthma exacerbation [3]. Similarly, glucocorticoids reduce airway NO during CPB [4]. Massaro et al. [21] also found extremely low (7 ± 1.2 ppb) NO concentrations sampled from the tracheal carina in intubated normal subjects during a breath-hold, while a group with inflammatory lung disease (asthma) had significantly elevated (40.5 ± 5.6 ppb) lower airway concentrations. These results [21] are consistent with the data reported in this study.

Cardiopulmonary bypass induces a systemic inflammatory response that is similar in many respects to
sepsis [4]. The higher endogenous concentrations of NO formed by cytokine activation of iNOS [7] may cause lung [19,20] and myocardial [22] reoxygenation (reperfusion) injury after CPB. Ruvolo et al. [8] found plasma nitrite concentration (a measurable breakdown product of NO) to rise during and after CPB, evidence (reperfusion) injury after CPB. Ruvolo et al. plasma nitrite concentration (a measurable breakdown of increased endogenous NO production during and immediately after CPB

Cardiopulmonary bypass is characterized by elevated ketones) [26]. Other serine protease inhibitors (chloromethylketones) [23] and aprotinin [24] inhibit cytokine-induced iNOS expression in vitro. Glucocorticoids, like aprotinin, inhibit cytokine-induced iNOS expression [25]. Transcription of iNOS is regulated by the nuclear regulatory protein, nuclear factor κB (NFκB) [26]. However, in order to exert its effects inside the nucleus, NFκB in the cytoplasm must first be disassociated from its inhibitor by a serine protease [27]. Cardiopulmonary bypass is characterized by elevated plasma concentrations of serine proteases, including, among others, activated complement fragments [28]. These data suggest the potential for inhibition of NFκB disassociation from its inhibitor by aprotinin, a protease inhibitor [23,24], resulting in a decrease in iNOS transcription.

The importance of cytokine-induced iNOS expression in sepsis pathophysiology is shown by several studies demonstrating that selective iNOS inhibition reverses the systemic hypotension and vascular hyporeactivity to vasoconstrictor agents which characterizes the septic state [29–31]. Cytokine-induced iNOS expression is currently thought to be the primary mechanism of myocardial depression in sepsis [32], and is also thought to play a major role in chronic heart failure in such pathological states as valvular or ischaemic heart disease as well as dilated cardiomyopathy [33]. Finally, cytokine-induced iNOS expression resulting in increased endogenous NO production is thought to be a mechanism in cardiac allograft rejection and programmed cell (myocyte) death (apoptosis) post cardiac transplant [34]. Thus, efforts at reducing cytokine-induced iNOS expression and therefore endogenous NO production may be beneficial in many clinical scenarios, including during and after CPB [6,12].

In summary, this study demonstrates that aprotinin, in a dose-responsive manner, reduces NO production in vivo, and reduces cytokine-induced nitrite production by murine bronchial epithelial cells in vitro. Since increased airway NO is found in inflammatory lung diseases, like asthma, and anti-inflammatory therapy reduces these concentrations of airway NO, these data support the concept that aprotinin is anti-inflammatory during CPB. In addition, these data are consistent with the concept that airway NO may be a biomarker of pulmonary inflammation during CPB.

REFERENCES