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ADENOSINE 5'-MONOPHOSPHATE IN ASTHMA: GAS EXCHANGE AND SPUTUM CELLULAR RESPONSES (ERJ-01162-2007. REVISION 1)

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(Word Count: 2,930)

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***Running Head**:* AMP and MCh Challenges in Asthma

Key Words: Direct and Indirect Bronchial Challenges - Induced Sputum - Multiple Inert Gas Elimination Technique - Pulmonary Gas Exchange - Short-Acting Bronchodilators

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ABSTRACT *(Word Count 193)*

Adenosine 5'-monophosphate (AMP) bronchoprovocation could reproduce the lung function abnormalities spontaneously occurring during acute asthma and detect peripheral airway inflammation better than direct bronchoconstrictive agents. Pulmonary gas exchange disturbances may reflect changes in small airways related to airway inflammation rather than bronchoconstriction alone.

We investigated whether AMP induces more ventilation-perfusion (V_A/Q) imbalance than methacholine (MCh) at equivalent degree of bronchoconstriction with and without salbutamol pre-medication. We studied 36 asthmatics in three randomised, double-blinded, cross-over studies: before and after AMP/MCh (Study-1); and before and 30 min after salbutamol/placebo, followed by AMP (Study-2)/MCh (Study-3) challenge. Sputum was collected before and 4 h post-challenge.

Compared to MCh, AMP provoked similar pulmonary gas exchange abnormalities at an equivalent degree of intense bronchoconstriction (FEV_1 fall, range 28-44%). While salbutamol blocked AMP/MCh-induced bronchoconstriction, PaO_2 and $AaPO_2$ disturbances were partially blocked (AMP, by 46% and 58%; MCh, by 42% and 57%, respectively). Compared to MCh, AMP increased neutrophils (from 28±17% to 38±16%, $p<0.05$) but not after salbutamol pre-treatment.

Both AMP and MCh induce similar peripheral airway dysfunction. The fully inhibited AMP-induced neutrophilia with residual hypoxaemia observed after salbutamol is likely related to beta-agonists acting on both bronchial and pulmonary circulations.

INTRODUCTION

Adenosine 5'-monophosphate (AMP) is a potent pharmacologic agent increasingly used in indirect bronchial challenge tests in patients with asthma. AMP once inhaled rapidly converts to adenosine by the enzyme 5'-nucleotidase and adenosine is a natural signaling nucleoside and mediator of airway inflammation that induces bronchoconstriction, most likely due to the release of inflammatory mediators from mast cells. However, it has also been postulated that adenosine modulates the function of many other cells involved in bronchial hyperresponsivenes and inflammation, such as neutrophils, eosinophils, lymphocytes, and macrophages (1). Moreover, it is suggested that AMP induces bronchoconstriction through the activation of inflammatory mechanisms at the level of bronchial surface and also through local or central neuronal reflexes (1). It is considered that bronchial challenges with direct agents, such as methacholine (MCh), assess the response to the agent acting directly on receptors while causing airway smooth muscle contraction; by contrast, bronchial challenges with indirect agents, such as AMP, assess the responses to endogenously released substances from resident inflammatory cells, hence reflecting the presence and severity of airway inflammation (2). Moreover, PC_{20} AMP is more closely associated with airway inflammation in asthma than is PC_{20} MCh (3) suggesting that PC_{20} AMP is more sensitive to airway inflammation since it shows a greater response to corticosteroids than does PC_{20} MCh (4). We hypothesised that bronchial challenge with AMP in patients with mild asthma, in addition to provoke bronchoconstriction in larger airways, could orchestrate inflammatory events and peripheral airway dysfunction resulting predominantly in pulmonary gas exchange disturbances. Compared with MCh, bronchial challenge with AMP could evoke more closely the gas exchange abnormalities spontaneously occurring during acute severe asthma. Previous studies in patients with asthma under different clinical conditions have consistently shown the compelling evidence of a poor correlation between the behaviour of reduced

maximal expiratory airflow rates and pulmonary gas exchange abnormalities, namely arterial hypoxaemia and its major intrapulmonary determinant, ventilation-perfusion (V_A/Q) imbalance (5,6). Conceivably, these findings concur with the hypothesis that decreased spirometric indices reflect reduction of airway calibre in larger and middle-sized bronchi, whereas pulmonary gas exchange disturbances predominantly relate to structural changes in distal small airways which could be more related to airway inflammation rather than to bronchoconstriction alone (6). Notwithstanding, a cause and effect relationship will be very difficult to establish in humans. Salbutamol, an inhaled short-acting beta-adrenergic agonist, inhibits PAF- and LTD_4-induced increased airway resistance, sputum and peripheral blood abnormal cellularity, and gas exchange defects in asthmatics (7-9). We postulated that these effects of salbutamol could be related to an inhibition of capillary endothelial constriction in the bronchial microcirculation (10) without necessarily reflecting its potent relaxant effect on airway smooth muscle.

The first aim of our study was to investigate whether AMP bronchoprovocation in patients with asthma could induce more V_A/Q imbalance, as a marker of predominant peripheral airway inflammation, than MCh challenge while provoking similar intense bronchoconstriction. A secondary endpoint of our study was to assess whether salbutamol could inhibit AMP-induced bronchoconstriction and arterial oxygenation defects while modulating the airway inflammatory cellular response. To date no data are available in the literature regarding the pulmonary gas exchange response to AMP challenge in asthma.

METHODS

Patients. 36 non-smoking patients with stable intermittent asthma (26±1 yrs; FEV_1, 91±4%; 17 females) were included. For inclusion, patients were required to have an FEV_1 > 70% predicted and > 1.5 L and a decrease in FEV_1 > 20% from baseline after a standardised AMP or MCh bronchial challenge. All patients were on rescue therapy with a short-acting inhaled β_2-agonist and four were using inhaled corticosteroids (one patient, 800 µg/d budesonide, and two patients, 640/18 µg/d budesonide/formoterol in Study-1 and one patient with the same combined therapy in Study-2). None were taking systemic glucocorticosteroids in the previous 3 months. The study was approved by the Ethics Committee of Hospital Clínic (Protocol # 02-0239: *Agencia Española del Medicamento*) and all the patients gave written informed consent.

Design. Three sequential studies (n = 12 patients, each) in a randomised, double-blinded, cross-over manner were designed: (a) to examine the effects of AMP and MCh on airway caliber, gas exchange and airway inflammation (Study-1), and (b) and (c) to analyse the role of pre-treatment with salbutamol in influencing AMP (Study-2) and MCh (Study-3) bronchial challenge.

Study-1: On the 1st visit, clinical evaluation, spirometry and MCh bronchial challenge were performed. One week later (2nd visit), induced sputum was obtained as a baseline assessment. On the 3rd visit the patients were randomised to AMP or MCh challenge and on the 4th visit to the alternative agent, one week apart. Dose-response challenges were performed until at least a 30% fall in FEV_1 from baseline. All sets of measurements including the multiple inert gas elimination technique (MIGET) and respiratory arterial blood gases were performed before (baseline), and then after each challenge, at 5, 15 and 45 min. Sputum was induced at 240 min after challenge.

Study-2: On the 1st visit, clinical evaluation, spirometry and AMP bronchial challenge were performed. On the 2nd visit, induced sputum was obtained as a baseline assessment. On the 3rd and 4th visits (one week apart) patients were challenged with AMP after randomization to inhaled salbutamol (400 μg) or placebo (lactose). All but MIGET sets of measurements of Study-1 were performed at baseline (B0), 30 min after salbutamol/placebo administration (B1), and then after AMP, at 5, 15 and 45 min. Sputum was induced at 240 min after challenge.

Study-3: This study followed exactly the same design of Study-2 but using MCh instead of AMP.

Measurements. FEV_1 and respiratory system resistance (Rrs), PaO_2, $PaCO_2$, oxygen consumption (VO_2), carbon dioxide production (VCO_2), and pH were measured; the alveolar-arterial PO_2 difference ($AaPO_2$) was calculated according to the alveolar gas equation using the measured respiratory exchange ratio. In Study-1 (AMP-MCh), MIGET was used to estimate the distribution of V_A/Q ratios without sampling mixed venous inert gases (11). Cardiac output (Q_T) was measured by dye solution technique using a 5 mL bolus of indocyanine green. After ensuring steady-state conditions, a set of duplicate measurements for each variable was obtained at each time point.

Fresh samples of induced sputum were processed before and 4h after each bronchial challenge (12). Concentrations of Interleukin-8 (IL-8) in Study-1, -2 and -3, and IL-2, IL-4, IL-10 and interferon-gamma (IFN-γ) in Study-2 and -3 were measured in sputum supernatant. Paired measurements of induced sputum were completed in Study-1 and -2 (10 patients each).

Statistical Analysis. Results are expressed as mean ± SE or 95% confidence interval (CI). PC_{25} for AMP and MCh were derived by linear interpolation from the log-cumulated dose-response curve and its geometrical mean was calculated on log-transformed raw data. In the three studies, the effects of AMP and MCh challenges with or without salbutamol/placebo

pre-medication on the different end-point variables were assessed by a two-way repeated analysis of variance (ANOVA). In Study-2 and Study-3, B1 was used as baseline. Whenever there were significant differences, *post-hoc* comparisons at each time point were performed using paired t-test. In addition, paired t-tests and Pearson's correlation were also used when necessary. Statistical significance was set at $p < 0.05$ in all instances.

RESULTS

Baseline. Patients had normal FEV_1 and arterial blood gases and mild increases in Rrs in all three studies without significant differences between them (Table 1). In Study-1, the dispersion of pulmonary blood flow (Log SDQ) (AMP range, 0.33-0.71; MCh range, 0.32-0.58) (normal ≤ 0.60) and that of alveolar ventilation (Log SDV) (AMP range, 0.33-0.67; MCh range, 0.32-0.58) (normal ≤ 0.65) (13) were narrowly unimodal and an overall index of V_A/Q heterogeneity (DISP R-E*) (AMP ranges, 1.03-6.21; MCh range, 0.91-3.99) (normal values ≤ 3.0) (14) was mildly increased. In Study-2, FEV_1 increased (from 3.5±0.2 to 3.9±0.2 L, $p<0.001$) and Rrs decreased (4.3±0.3 to 3.5±0.2 $cmH_2O.L^{-1}.s$, $p<0.02$) from B0 (before) to B1 (after) in the salbutamol arm; similarly, in Study-3, FEV_1 increased (from 3.3±0.2 to 3.7±0.3 L) while Rrs decreased (3.9±0.4 to 2.9±0.4 $cmH_2O.L^{-1}.s$) ($p<0.02$ each) from B0 to B1 after pre-treatment with salbutamol. No other significant differences in PaO_2 and $AaPO_2$ were observed between measurements carried out before and after salbutamol/placebo pre-treatment (Figure 1).

Study-1: AMP and MCh Responses. Five min after AMP challenge (mean cumulative doses, 6.7 mg), there were moderate-to-severe decreases in both FEV_1 and PaO_2 and increases in both Rrs and $AaPO_2$ that were not different from those shown after MCh-induced (mean cumulative doses, 0.15 mg) bronchoconstriction (Table 2). AMP- and MCh-induced decreases in PaO_2 were caused by mild-to-moderate V_A/Q inequalities, as reflected by increases of the same order of magnitude in both Log SDQ and DISP R-E* whereas Log SDV remained unchanged. Overall, V_A/Q distributions were broadly unimodal. By contrast, $PaCO_2$, pH, VO_2, VE, f, Ps, and HR, remained essentially unchanged after challenge. Cardiac output, compared to MCh challenge, increased after AMP bronchoconstriction at 5 (by 15%) and 15 min (by 11%) ($p<0.05$ each). All but Q_T abnormal variables showed a trend to recover at 15 and 45 min after AMP. Both FEV_1 and Rrs were mildly abnormal at the end of the study. The

residual sum of squares (RSS), the best descriptor of the quality of MIGET data, was within the expected limits (≤ 5.0) (13) (AMP, 2.1±0.3; MCh 2.4±0.4). Four hours after AMP inhalation, sputum neutrophils increased significantly ($p<0.05$) (Table 3). Two out of the three patients previously treated with inhaled glucocorticosteroids did not show a distinct neutrophils increase post-AMP challenge; in the third patient, sputum was not obtained. IL-8 showed a trend to decrease after AMP and MCh challenges compared to baseline values (Table 3). Baseline DISP R-E* and PaO_2 were inversely correlated before AMP challenge ($r = - 0.61$, $p<0.05$). Five min after MCh challenge, PaO_2 inversely correlated with Log SDQ ($r = - 0.93$, $p<0.01$) and Log SDV ($r = - 0.65$, $p<0.05$). There was a positive correlation between FEV_1 and PaO_2 changes at 5, 15 and 45 min after AMP ($r = 0.78$, $p<0.01$) and MCh ($r = 0.69$; $p <0.01$) challenge.

Study-2: AMP Challenge and Pre-treatment with Salbutamol. As compared with placebo, pre-treatment with salbutamol completely blocked the bronchoconstriction induced by AMP (mean cumulative doses, 3.6 mg) such that FEV_1 and Rrs remained unchanged at 5 min (Table 4; Figure 1); by contrast, AMP-induced changes in PaO_2 and $AaPO_2$ were partially blocked (by 46% and 58%, respectively) (Table 4; Figure 1), an inhibitory effect that persisted less intensely at 15 min ($p<0.05$ each); by 45 min, both PaO_2 and $AaPO_2$ showed a trend to return to baseline levels. Moreover, AMP-induced neutrophilia was significantly blocked by pre-medication with salbutamol. Compared to baseline, there were no significant differences in the supernatant concentration of IL-8 and IL-2 after AMP challenge in both arms (Table 3). IL-4, IL-10 and IFN-γ supernatant concentrations were not detected in more than 89% of samples (data not shown). The percentage of neutrophils at baseline inversely correlated with PaO_2 ($r = - 0.84$, $p<0.01$) while IL-2 positively correlated with the percentage of eosinophils at 5 min post-AMP challenge ($r = 0.87$, $p<0.01$). There was a close correlation between

decreased FEV_1 and PaO_2 changes at 5, 15 and 45 minutes after AMP challenge pre-treated with placebo ($r = 0.69$, $p<0.01$).

Study-3: MCh challenge and Pre-treatment with Salbutamol. As in Study-2, compared to placebo, salbutamol at 5 min inhibited completely MCh-induced bronchoconstriction but only partially PaO_2 (by 42%) and $AaPO_2$ (by 57%) abnormalities (Table 4; Figure 1), changes that persisted at 15 min ($p<0.05$ each); by 45 min, compared to placebo, PaO_2 and $AaPO_2$ changes still remained slightly different from placebo. No substantial changes were observed in sputum cells after pre-medication with salbutamol/placebo. Overall, IL-4, IL-10 and IFN-γ supernatant concentrations were not detected in more than 86% of samples (data not shown). There was a significant correlation between the time course of the FEV_1 fall (expressed as % change from baseline) and that of PaO_2 at 5, 15 and 45 min after challenge ($r = 0.70$; $p<0.01$). No differences were observed in the four principal variables (FEV_1, Rrs, PaO_2 and $PaCO_2$) between salbutamol/placebo arms when AMP (Study-2) and MCh (Study-3) challenges were compared.

DISCUSSION

Major Findings. Three were the principal novel findings in the present study in patients with stable mild asthma. Firstly, compared to MCh, AMP (Study-1) transiently provoked similar pulmonary gas exchange abnormalities at an equivalent degree of intense bronchoconstriction essentially characterised by moderate decreases in PaO_2 and increases in $AaPO_2$ due to the development of low V_A/Q ratio units as assessed by increases in Log SDQ (dispersion of blood flow). Similarly, we observed arterial blood gas abnormalities, although slightly less pronounced, when AMP and MCh bronchoprovocations were randomised to placebo (Study-2 and -3). Secondly, AMP challenge provoked mild sputum neutrophilia, an effect that was completely blocked by salbutamol pre-medication (Study-2). Thirdly, pre-treatment with salbutamol completely blocked AMP- and MCh-induced bronchoconstriction but only partially arterial blood gas disturbances (Study-2 and -3).

Pulmonary Gas Exchange Response to AMP and MCh challenge. The post-challenge pulmonary gas exchange defects resulting in moderate decreases in PaO_2 and increases in $AaPO_2$ due to mild-to-moderate V_A/Q imbalance did not differ after each bronchoconstrictive agent. However, the simultaneous increase in Q_T immediately after AMP, likely related to AMP-induced inotropism (15), should have increased mixed-venous oxygen content, hence increasing PaO_2 other things being equal (6). Alternatively, post-AMP increased Q_T should have facilitated further V_A/Q worsening by diverting more blood flow to low V_A/Q ratio units. The latter changes may have been offset by a simultaneous AMP-induced enhancement of hypoxic pulmonary vasoconstriction, hence reducing the deleterious impact of increased Q_T on V_A/Q imbalance.

These post-AMP gas exchange defects are akin to those observed following different types of direct, *i.e.* allergens (16), MCh and histamine (17), and leukotriene D_4 (LTD_4) (18), or indirect agents, *i.e.* platelet-activating factor (PAF) (19). Only, AMP-induced gas exchange

abnormalities differed from those provoked by both exercise and mannitol bronchoprovocation that affected not only the dispersion of blood flow (Log SDQ) but also that of alveolar ventilation (Log SDV) (20). Altogether these bronchial challenge-induced gas exchange findings indicate that all direct and most indirect agents provoke similar bronchoconstrictive responses irrespective of the initial biochemical and/or cellular pathway (21). Therefore, the present findings refute our hypothesis that AMP could produce more widespread airway inflammation, thus more V_A/Q imbalance than MCh. Instead, AMP-induced gas exchange abnormalities point to the view that the mechanisms of bronchial responsiveness are similarly heterogeneous in their topographical basis and distributed in both central and peripheral airways, a finding already observed after MCh and histamine challenge in mild asthma (17,18).

Induced Sputum Findings. There was a significant late increase in neutrophils in induced sputum after AMP inhalation, at variance with the predominant eosinophilia shown earlier after AMP challenge (22,23). This neutrophilia is however consistent with previous data observed in persistent asthma (24) and in a mouse AMP model (25). Our findings therefore provide the first evidence that AMP inhalation has a late effect on airway neutrophil migration in asthmatics. There is evidence that AMP challenge may provoke cellular chemoattraction within the airways through the release of a variety of inflammatory mediators from lung mast cells, namely LTB_4, IL-5, IL-8 and tumor necrosis factor-alpha (TNF-α), all chemoattractants for neutrophils (12,26). The percentage of neutrophils did not correlate with IL-8 in our study, possibly because the latter measurements were not sufficiently sensitive or other mediators and/or receptors not measured could have been involved.

Salbutamol Effects. Salbutamol exerted a complete bronchoprotective effect against AMP and MCh inhalation but only a partial inhibition on pulmonary gas exchange disturbances.

Our findings include a less intense inhibitory effect than those observed after PAF (27) or LTD_4 (7) in asthmatics pre-treated with salbutamol, in whom using a similar dosage salbutamol fully inhibited PAF- and LTD_4-induced increased systemic and serum cellular abnormalities. We hypothesised that the latter effects could be preferentially related to an inhibition of endothelial venoconstriction in the airway microcirculation and the subsequent release of mediators that induce abnormal vascular permeability, a mechanism that can be also invoked to explain the AMP-induced neutrophilia inhibition. Airway mucosal blood flow is increased in stable asthma compared to normal individuals and does not increase following a standard dose of salbutamol (28). The intriguing finding in our study is that we should have expected a complete inhibition of post-salbutamol gas exchange abnormalities akin to the full inhibitory cellular response. The finding of post-salbutamol residual mild gas exchange abnormalities in the absence of evident bronchoconstriction indicates some persistant V_A/Q imbalance in peripheral lung regions. This V_A/Q mismatching may be caused by beta-agonist-induced pulmonary vasodilatation or persistent small airway narrowing where bronchodilators are less influential. It is likely that the two latter mechanisms may coexist considering the degree of residual arterial hypoxaemia after salbutamol during AMP and MCh challenges. Notwithstanding, the potentially favoured central deposition of salbutamol (29) coupled with the contention that small airway inflammation is more significant in terms of the likelihood of these airways to become more obstructed than larger airways, due to the thickness of inflamed epithelium and/or mucus layer is proportionally more important in smaller airways, cannot be neglected.

Conclusions. Our findings indicate that both AMP and MCh provoke similar gas exchange abnormalities during intense bronchoconstriction; however, at variance with MCh, AMP induces a late sputum neutrophilic response. These findings suggest, firstly, that both bronchoconstrictive agents share a common mechanism of airway narrowing and, secondly,

that the initial pathways differentiating their direct or indirect effects may overlap if severe bronchoconstriction is reached. Salbutamol exerted a complete inhibition of AMP-induced bronchoconstriction and sputum neutrophilia but only a partial blockade of gas exchange abnormalities, hence indicating that short-acting beta-agonists may induce pulmonary vasodilatation possibly associated with incomplete reversion of small airway dysfunction.

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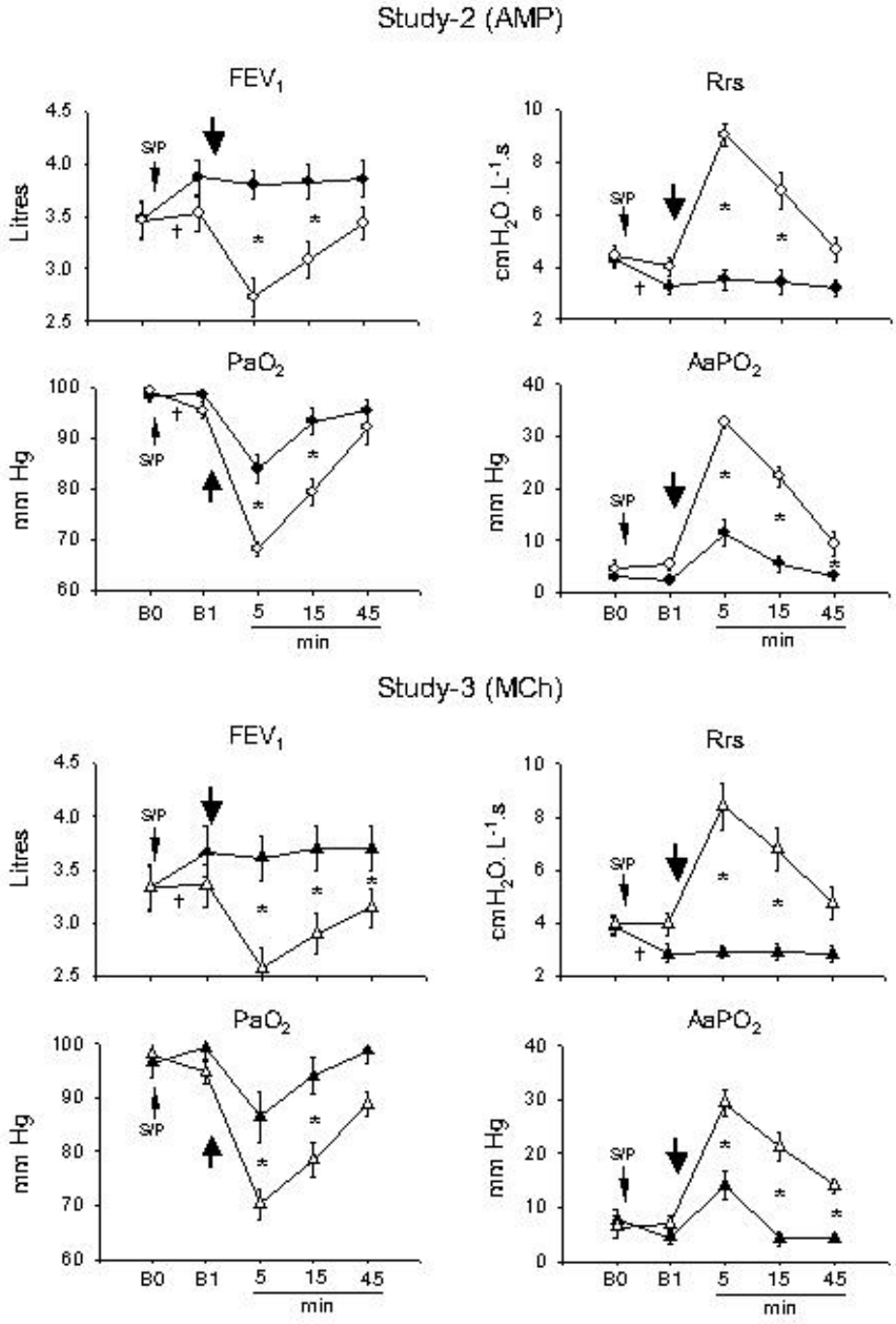
LEGEND TO FIGURE

FIGURE 1. Time courses (mean ± SE) for FEV_1, Rrs, PaO_2, and $AaPO_2$ measured at baseline (B0), 30 min after salbutamol (closed symbols)/placebo (open symbols) pre-treatment (B1), and at 5, 15 and 45 min after AMP (Study-2) and MCh (Study-3) bronchial challenge. Thin arrows represent salbutamol (S)/placebo (P) and bold arrows indicate AMP/MCh challenge. Asterisks: denote significant difference between S and P at each time point. (†): significantly different between B0 and B1 for S or P.


Study-2 (AMP)
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mm Hg
AaPO2
mm Hg
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B1
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15
45
min
Study-3 (MCh)
FEV1
Litres
Rrs
cmH2O.L-1.s
PaO2
mm Hg
AaPO2
mm Hg

FIGURE 1