INCREASED ARGINASE UNDERLIES INFLAMMATION AND REMODELLING IN A GUINEA PIG MODEL OF COPD

Tonio Pera, Annet Zuidhof, Marieke Smit, Mark Menzen, Theo Klein, Gunnar Flik, Johan Zaagsma, Herman Meurs, Harm Maarsingh

Submitted (2011)
Abstract

Airway inflammation and remodelling are major features of COPD, while pulmonary hypertension is a common comorbidity associated with a poor disease prognosis. Recent studies in animal models have indicated that increased arginase activity contributes to features of asthma, including allergen-induced airway inflammation and remodelling. Although cigarette smoke and lipopolysaccharide (LPS), major risk factors of COPD, may increase arginase expression, the role of arginase in COPD is unknown. This study aimed to investigate the role of arginase in pulmonary inflammation and remodelling using an animal model of COPD. Guinea pigs were instilled intranasally with LPS or saline twice weekly for 12 weeks and pretreated by inhalation of the arginase inhibitor 2(S)-amino-6-boronoctanoic acid (ABH) or vehicle.

Repeated LPS exposure increased lung arginase activity, resulting in increased L-ornithine/L-arginine and L-ornithine/L-citrulline ratios. Both ratios were reversed by ABH. ABH inhibited the LPS-induced increases in pulmonary IL-8, neutrophils and goblet cells as well as airway fibrosis. Remarkably, LPS-induced right ventricular hypertrophy, indicative of pulmonary hypertension, was prevented by ABH.

In conclusion, increased arginase activity contributes to pulmonary inflammation, airway remodelling and right ventricular hypertrophy in a guinea pig model of COPD, indicating therapeutic potential for arginase inhibitors in this disease.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by a progressive decline in lung function and airflow limitation that is not fully reversible. Chronic inflammation, characterized by increased numbers of neutrophils, macrophages, CD8+ and CD4+ T lymphocytes and B cells in the lung, could contribute to structural changes underlying the airflow limitation, including emphysema and airway remodeling (1). Airway remodeling in COPD is predominantly characterized by mucus cell hyperplasia and peribronchiolar fibrosis (2). In addition, pulmonary hypertension, a comorbidity present in a large proportion of COPD patients, may lead to right ventricular hypertrophy and pulmonary vascular remodeling (3-5).
Recent studies in animal models (6-11) and in patients (8, 10, 12-16) have indicated a major role for increased arginase activity in the pathophysiology of asthma. Increased activity of arginase, which converts L-arginine to L-ornithine and urea, decreases the L-arginine bioavailability to constitutive and inducible isoforms of nitric oxide synthase (NOS) in the airways. This results in decreased production of bronchodilatory NO as well as increased synthesis of proinflammatory and procontractile peroxynitrite, which contribute to the development of allergen-induced airway hyperresponsiveness (AHR) (6, 9, 11). Treatment with inhaled arginase inhibitors strongly protected against allergen-induced airway obstruction, AHR and airway inflammation in guinea pig (7) and mouse (14, 17) models of acute allergic asthma in vivo. Using repeatedly allergen-challenged guinea pigs, we recently demonstrated that increased arginase activity also has a major role in airway remodeling in chronic asthma, as indicated by effective inhibition of these features by the inhaled arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) (18). In addition to changes in NO metabolism, this may involve increased production of L-ornithine downstream products such as polyamines and L-proline, that cause cell proliferation and collagen synthesis, respectively (10).

Although several studies have revealed the important role of arginase, particularly arginase I, in the pathophysiology of asthma, little is known about its role in COPD (8). However, increased arginase activity was already demonstrated in the late 1970s in sputum from patients with chronic bronchitis (19, 20) and more recently in bronchoalveolar lavage (BAL) fluid (21) and platelets (22) from COPD patients. Interestingly, cigarette smoke has been shown to induce arginase I expression in rat lung (23) and in airways from patients with mild asthma (24). High constitutive expression of arginase I has been demonstrated in azurophilic granules from human neutrophils (25), which are known to be released in COPD (26). Increased arginase activity and decreased NO synthesis have also been implicated in pulmonary arterial hypertension (26), a comorbidity of COPD.

In the present study we investigated the role of arginase in features of pulmonary inflammation, airway remodeling and pulmonary hypertension in a guinea pig model of lipopolysaccharide (LPS)-induced COPD (Chapter 5). LPS, a contaminant of cigarette smoke and environmental pollution, has been implicated in the development of COPD (27-31), and LPS exposure of experimental animals may induce various features of this disease, including inflammation, airway remodeling and emphysema (32-34). In addition, LPS has been shown to induce increased arginase expression in alveolar macrophages (35, 36) and lung tissue (37, 38).
Materials and Methods

Animals
Outbred, male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, United Kingdom) weighing 350-400 g were used. All protocols were approved by the University of Groningen Committee for Animal Experimentation.

Experimental protocol
Guinea pigs were challenged by intranasal instillation with either 200 μl LPS (5 mg/ml in saline) or 200 μl saline twice weekly, for 12 consecutive weeks (Chapter 5). Thirty min before each instillation, animals received a nebulised dose of the arginase inhibitor ABH in phosphate-buffered saline (PBS) (25 mM nebulizer concentration, 15 min) or PBS (15 min), using a DeVilbiss nebulizer (39). Twenty-four h after the last instillation, the guinea pigs were humanely euthanized by experimental concussion, followed by rapid exsanguination. Heart and lungs were immediately resected and kept in Krebs-Henseleit buffer or on ice, respectively, for further processing.

Arginase activity assay
Arginase activity, expressed as pmol urea produced per mg protein per min, was determined in lung homogenates, by measuring the conversion of [14C]-L-arginine to [14C]-urea at 37°C (9).

Amino acid quantification
Frozen lung tissue was homogenized in Tris-HCl buffer (50 mM Tris-HCl, 150 mM NaCl; pH 7.5) and centrifuged (12 000 x g; 20 min; 4°C) to remove insoluble material. In the supernatants, concentrations of the amino acids L-ornithine, L-arginine and L-citrulline were determined using high performance liquid chromatography followed by tandem mass spectrometry (HPLC-MS/MS) as described recently (18).

Interleukin-8 determination
Interleukin-8 (IL-8) was determined in lung homogenates using an enzyme-linked immunosorbent assay (ELISA) for guinea pig IL-8 according to manufacturer’s instructions (Cusabio Biotech, Wuhan, China).

Tissue analysis
Transverse frozen cross-sections (4 μm) of the middle right lung lobe were used for histological and immunohistochemical analyses. Neutrophils were identified by staining sections for TNAP (tissue non-specific alkaline phosphatase activity)
Arginase in inflammation and remodelling in COPD

(40). MUC5A/C antibody (Neomarkers; Fremont, CA, USA) was used to identify MUC5A/C-expressing goblet cells (41). Sections were counterstained with haematoxylin. Airways within sections were digitally photographed (40-200x magnification) and classified as cartilaginous or non-cartilaginous. Measurements were performed using ImageJ or NIS (Nikon) quantification software. Neutrophils in the airway adventitia and sub-mucosa were expressed as number of positively stained cells/mm basement membrane length (34). Parenchymal neutrophils were expressed as a percentage of total cell counts (34). MUC5A/C-positive cells in the epithelium were expressed as number of cells/mm basement membrane length (Chapter 5).

The upper right lung lobe was inflated and fixed with formalin at 25 cm H₂O constant pressure for 24 h, and embedded in paraffin. For evaluation of pulmonary vascular dimensions, sections (4 µm) were stained with Weigert’s elastin (resorcin/fuchsin) and Van Gieson stain (42). Pulmonary vessel dimensions were determined as described in Chapter 5. For evaluation of airway wall collagen, sections were stained with Sirius Red and counterstained with haematoxylin. The positively stained area in the airway wall, from the adventitial border to the basement membrane, of non-cartilaginous airways was determined as described in Chapter 5. The airway wall collagen area was normalized to the square of the basement membrane length.

To evaluate right ventricular hypertrophy, Fulton’s index, i.e. the ratio of the right ventricle weight and the sum of the septum and left ventricle weights, was determined.

**Hydroxyproline assay**

Lungs were analysed for hydroxyproline as an estimate of collagen content, using chloramine T and Erlich’s solution (Chapter 5).

**Statistical analysis**

Data are presented as mean ± SEM. Statistical differences between means were calculated using an unpaired two-tailed Student’s t-test or one-way ANOVA, followed by a Bonferroni or Newman Keuls multiple comparison test, as appropriate. Differences were considered significant when $P<0.05$. 

147
Chapter 7

**Results**

**Arginase activity and amino acid concentrations in the lung**

LPS induced a 2.2-fold increase in arginase activity in lung homogenates *ex vivo* (Figure 1A). Both in LPS- and in saline-challenged animals *in vivo* treatment with inhaled ABH did not significantly change the arginase activity measured *ex vivo* (Figure 1A). Repeated LPS challenge also increased the L-ornithine/L-arginine and L-ornithine/L-citrulline ratio’s in the lung (Figure 1B&C), indicating that the endogenous arginase activity is increased and that the balance between arginase and NOS activity is shifted towards arginase. ABH treatment did not affect the amino acid ratios in saline-challenged animals. However, in the LPS-challenged animals, ABH treatment reduced both the L-ornithine/L-arginine ratio and the L-ornithine/L-citrulline ratio to levels below those observed in the PBS-treated, saline-challenged animals (Figure 1B&C). Collectively, these data indicate that LPS instillation induces increased arginase activity in the lung, which is inhibited by ABH *in vivo*.

**Table 1:** Levels of L-arginine, L-ornithine and L-citrulline in lung homogenates of guinea pigs following repeated saline or LPS challenge and treatment with either inhaled PBS or ABH.

<table>
<thead>
<tr>
<th></th>
<th>PBS-treated</th>
<th>ABH-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline challenged</td>
<td>LPS challenged</td>
</tr>
<tr>
<td>L-Arginine (μmol/mg protein)</td>
<td>3.77±1.02</td>
<td>3.36±0.66</td>
</tr>
<tr>
<td></td>
<td>LPS challenged</td>
<td>3.85±0.42</td>
</tr>
<tr>
<td>L-Orrnithine (μmol/mg protein)</td>
<td>3.99±1.08</td>
<td>2.66±0.70</td>
</tr>
<tr>
<td></td>
<td>Saline challenged</td>
<td>LPS challenged</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.13±0.40</td>
</tr>
<tr>
<td>L-Citrulline (μmol/mg protein)</td>
<td>2.54±0.51</td>
<td>1.92±0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.92±0.12</td>
</tr>
</tbody>
</table>

Data represent means ± SEM of 5-8 experiments. *P<0.05 vs saline-challenged control; †P<0.05 vs LPS-challenged control.
Figure 1: Arginase activity (A) and L-ornithine/L-arginine (B) and L-ornithine/L-citrulline (C) ratios in lung homogenates from guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-8 experiments performed in duplicate. *P<0.05 vs saline-challenged control; †††P<0.001 vs LPS-challenged control.

Inflammation

Neutrophils are a major inflammatory cell type involved in COPD pathogenesis and are a rich source of arginase (25). Repeated LPS instillation increased the neutrophil number in both cartilaginous (2.9-fold) and non-cartilaginous (3.2-fold) airways as well as in the parenchyma (2.0-fold) (Figure 2). ABH treatment reduced the neutrophil numbers in these compartments by 83%, 60% and 56%, respectively (Figure 2). ABH treatment did not affect neutrophil numbers in saline-challenged animals. In order to assess potential mechanisms involved in arginase-induced neutrophilia in the LPS-challenged animals, we determined
levels of the neutrophil chemoattractant IL-8 in lung homogenates. Figure 3 indicates that the induction of neutrophil infiltration by repeated LPS instillation is associated with a significant increase of IL-8 in the lung, which was fully inhibited by inhalation of ABH. As with neutrophilia, no effects were observed in saline-challenged animals (Figure 3). These data indicate that LPS-induced arginase activity contributes to neutrophilia by increasing IL-8 levels.

**Figure 2:** Neutrophil numbers in the cartilaginous (A) and non-cartilaginous (B) airways and in lung parenchyma (C) of guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-8 experiments. *P<0.05; **P<0.01; ***P<0.001 vs saline-challenged control; †P<0.05; ††P<0.01 vs LPS-challenged control.
Arginase in inflammation and remodelling in COPD

Figure 3: IL-8 levels in lung homogenates from guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-8 experiments performed in duplicate. **P<0.01 vs saline-challenged control; †††P<0.001 vs LPS-challenged control.

MUC5A/C expression
Repeated LPS instillation induced a significant 2.2-fold increase in the number of MUC5AC-positive cells in the epithelium of cartilaginous airways (Figure 4), indicating mucus hypersecretion. ABH treatment fully inhibited the LPS-induced MUC5A/C expression, whereas it had no effect in saline-challenged animals (Figure 4).

Figure 4: MUC5A/C-positive goblet cell number in intrapulmonary cartilaginous airways of guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-7 experiments. **P<0.01 vs saline-challenged control; ††P<0.001 vs LPS-challenged control.
Airway fibrosis
To evaluate fibrotic changes, lungs were analysed for hydroxyproline as an estimate of collagen content. Repeated LPS instillation induced a significant 1.7-fold increase in total lung hydroxyproline content (Figure 5A). ABH treatment inhibited the LPS-induced increase in hydroxyproline by 75%, whereas it had no effect on the hydroxyproline content in saline-challenged animals (Figure 5A).
To assess changes in collagen deposition in the airway compartment, Sirius Red staining was evaluated in the airway wall of non-cartilaginous airways. Similar to the increase in hydroxyproline content, LPS induced a 1.9-fold increase in airway wall collagen content (Figure 5B). ABH fully inhibited the LPS-induced collagen deposition in the airway wall, whereas it did not affect the collagen content in the airway wall of saline-challenged animals (Figure 5B).

![Graph A](image1)
![Graph B](image2)

**Figure 5**: Whole lung hydroxyproline content (A) and collagen content in the airway wall (Sirius red; B) in guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-8 experiments. Hydroxyproline determinations were performed in triplicate and 2 to 6 airways were analysed for each animal for the Sirius red staining. *P<0.05, **P<0.01 vs saline-challenged control; †P<0.05, ††P<0.01 vs LPS-challenged control.

Right ventricular hypertrophy
Repeated LPS challenge induced right ventricular hypertrophy as indicated by a significant 1.4-fold increase in Fulton index (Figure 6). ABH treatment fully inhibited the LPS-induced right ventricular hypertrophy, whereas ABH had no effect in saline-challenged animals (Figure 6).
Arginase in inflammation and remodelling in COPD

Pulmonary vessel wall dimensions
To evaluate pulmonary vessel wall dimensions, pulmonary artery medial area and pulmonary arteriole wall area were determined in formalin-fixed, paraffin-embedded guinea pig lung sections stained with Weigert’s elastin and Van Gieson stain. Neither repeated LPS instillation nor ABH treatment affected the medial area of pulmonary arteries or wall area of pulmonary arterioles (Figure 7). In addition, there was no evidence of intimal proliferation in the pulmonary vessels of either classification.

Figure 6: Fulton’s index in guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 4-8 experiments. **P<0.01 vs saline-challenged control; ††P<0.01 vs LPS-challenged control.

Figure 7: Pulmonary artery medial area (A) and pulmonary arteriole wall area (B) in guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 4-8 experiments for the arteries and 3-4 experiments for the arterioles.
Discussion

This is the first study to demonstrate the effectiveness of an (inhaled) arginase inhibitor in preventing indices of pulmonary inflammation, airway remodeling and pulmonary hypertension in an animal model of COPD. Thus, inhaled ABH protected against neutrophil infiltration, mucus hypersecretion and airway fibrosis induced by repeated intranasal LPS instillation in guinea pigs. In addition, repeated LPS challenge induced right ventricular hypertrophy, which was similarly inhibited by inhalation of the arginase inhibitor.

It was found that repeated LPS challenge in vivo increased arginase activity in guinea pig lung homogenates determined ex vivo. This presumably reflects increased arginase expression induced by the LPS challenge, as arginase is a constitutively active enzyme. Unfortunately, due to lack of specific antibodies against (subtypes of) guinea pig arginase it was not possible to determine arginase protein expression in a direct manner; however, increased arginase gene expression in the lung induced by inhalation of LPS has previously been observed in mice (37, 38). The lack of effect of ABH inhalation on the induction of increased arginase activity by LPS as measured ex vivo (ABH not being present in the assay) suggests that arginase is not involved in the regulation of its own expression. This is in contrast with allergen-induced increase in arginase activity, that can be inhibited by arginase inhibitors (17, 68). This is presumably due to potentiation of allergen-induced IL-13 production by constitutive arginase activity present in the airways, which in turn may enhance arginase expression (10). The LPS-induced increase in arginase activity determined ex vivo was reflected by increased L-ornithine/L-arginine and L-ornithine/L-citrulline ratio’s in the lung. The LPS-induced increase in L-ornithine/L-citrulline ratio indicates that the increased arginase activity competes with NOS for L-arginine. Treatment with inhaled ABH prevented the enhanced L-ornithine/L-arginine ratio induced by LPS, indicating that endogenous arginase activity was indeed inhibited by inhaled ABH. Moreover, the reduction in the L-ornithine/L-citrulline ratio by ABH indicates restoration of NOS activity by the arginase inhibitor. Interestingly, ABH treatment of the LPS-challenged animals resulted in attenuation of both ratios below the levels observed for saline-challenged animals. The reduced L-ornithine/L-citrulline ratio below baseline might be explained by LPS-induced iNOS activity, which results in increased L-citrulline production, as also reflected by the trend towards an increase of the L-citrulline concentration in the lungs of ABH-treated, LPS-challenged animals (Table 1). Indeed, induction of iNOS by LPS is well established (43). Increased expression of iNOS could also account for the decreased L-ornithine/L-arginine ratio, as recycling of L-citrulline is an
Arginase in inflammation and remodelling in COPD

important source of L-arginine, under inflammatory conditions (44, 45). This is also supported by the observation that argininosuccinate synthetase, an enzyme which plays a key role in the conversion of L-citrulline to L-arginine, is upregulated in the lung by LPS treatment in vivo (38, 46). Taken together, our findings indicate that LPS induces increased arginase activity in the lung in vivo, which is inhibited by inhaled ABH, thereby favoring NOS activity and increasing NO production. Since NO has anti-inflammatory and anti-fibrotic actions (47), such a mechanism may well be involved in the inhibition of LPS-induced neutrophil influx, collagen synthesis and mucus production by the arginase inhibitor, as is also discussed below.

Increased arginase activity has previously been found in BAL fluid (21) and platelets (22) of COPD patients. In addition to LPS, arginase expression is also induced by cigarette smoke as shown in rat lung (23) and rabbit cavernous tissue (48). Moreover, arginase expression in the airways is further increased in patients with mild asthma who smoke, compared to non-smoking asthmatics (24).

Neutrophils are involved in the pathogenesis of COPD. Pulmonary neutrophils are increased in COPD and correlations between airway neutrophil numbers and COPD severity have been found (26). ABH inhalation strongly inhibited LPS-induced neutrophilia in our model, indicating that induction of arginase by LPS importantly contributes to the neutrophilic inflammation. To investigate possible mechanisms underlying this anti-inflammatory effect of ABH, we determined concentrations of the major neutrophil-attracting chemokine IL-8 in whole lung homogenates. LPS-induced neutrophilia was associated with an increased IL-8 in the lung, while neutrophil influx and increase in IL-8 were both inhibited by ABH, suggesting that increased arginase activity may contribute to neutrophilia by increasing IL-8 levels in the lung.

One of the mechanisms underlying enhanced IL-8 production and airway inflammation by arginase might be via promoting NF-κB by attenuating the synthesis of NO, which inhibits this process via nitrosylation of the transcription factor (49). In addition, increased arginase activity causes uncoupling of iNOS and subsequent production of the pro-inflammatory oxidant species peroxynitrite (8), which induces IL-8 expression in various cell types (50, 51). Accordingly, breakdown of this oxidant reduces smoke-induced IL-8 levels in sheep lung (52).
Mucus hypersecretion contributes to airflow limitation in COPD. Increased MUC5AC expression is observed in the airway epithelium of COPD patients and can be induced by cigarette smoke and LPS, as well as by neutrophil elastase and peroxynitrite (53). ABH fully inhibited the LPS-induced MUC5AC expression in the guinea pig airway epithelium, indicating a major role for increased arginase activity in this process. The effect of ABH may be the result of the inhibition of IL-8 production and airway neutrophilia, which may both contribute to increased MUC5AC expression (54, 55). Moreover, ABH could decrease the LPS-induced MUC5AC expression by inhibiting peroxynitrite formation and restoring NO production (56).

Airway fibrosis is a characteristic feature of COPD, which contributes to airway wall thickening and airflow limitation (57). Previous studies have shown that increased arginase expression contributes to bleomycin-induced lung fibrosis in mice (58), lung allograft fibrosis in rats (59) and repeated allergen challenge-induced fibrosis in guinea pigs (68). The present study indicates that increased arginase activity also contributes to LPS-induced fibrosis in the lung, particularly in the airway wall. This may involve increased production of L-ornithine and its downstream product L-proline, which is a precursor of collagen (60). In accordance, TGF-β, a major pro-fibrotic factor, has been shown to induce arginase activity in the rat lung and fibroblasts (59) and TGF-β-induced collagen synthesis was reduced by inhibitors of arginase in lung fibroblasts of rats and mice (61, 62). In addition, the inhibition of fibrosis by ABH may also be due to the increased production of NO and decreased formation of peroxynitrite (63, 64).

Our data show that repeated LPS-challenge induces right ventricular hypertrophy, a feature of pulmonary hypertension, a known co-morbidity of COPD (3). The LPS-induced right ventricular hypertrophy was prevented by ABH. Our data therefore suggest that repeated LPS challenge results in pulmonary hypertension via induction of arginase. Pulmonary hypertension and right ventricular hypertrophy may result from (a combination of) vascular remodeling and functional changes in the vessel wall, both leading to increased resistance in the pulmonary vasculature (3). In our model, we did not observe changes in pulmonary vessel dimensions after repeated LPS instillation or by treatment with inhaled ABH, suggesting that increased resistance in the pulmonary vessels and subsequent right ventricular hypertrophy in this model are due to exaggerated constriction of the vessels rather than remodeling. In this respect, endothelial dysfunction caused by reduced activity of eNOS has been proposed as a potential mechanism (65). Indeed, pulmonary hypertension has
been associated with reduced L-arginine and NO levels (66), whereas inhalation of NO and oral therapy with L-arginine decrease pulmonary arterial pressure in primary or secondary pulmonary hypertension (67). Increased consumption of L-arginine by enhanced arginase II expression and activity in the endothelium has been shown to contribute to the reduced L-arginine and NO levels (66, 67). In addition, hypoxia, which is considered to play a major role in COPD-related pulmonary hypertension, upregulates arginase in human lung microvascular endothelial cells (68). Our data would suggest that increased arginase activity may contribute to pulmonary hypertension and right ventricular hypertrophy in COPD, possibly by inducing endothelial dysfunction, and that this process can be effectively targeted by inhalation of arginase inhibitors.

In conclusion, our study demonstrates that increased arginase activity plays a major role in pulmonary inflammation, airway remodeling and right ventricular hypertrophy in a guinea pig model of COPD and that arginase inhibitors may have therapeutic potential in the treatment of this disease.

Acknowledgements

This study was supported by MSD, Oss, The Netherlands and The Graduate School of Behavioral and Cognitive Neurosciences, University of Groningen, Groningen, The Netherlands

References

3. MacNee W. Right heart function in COPD. *Semin Respir Crit Care Med* 2010;31:295-312.


