Modulation of Cutaneous Inflammation by Angiotensin-Converting Enzyme

Thomas E. Scholzen, Sonja Ständer, Helge Riemann, Thomas Brzoska and Thomas A. Luger

*J Immunol* 2003; 170:3866-3873; doi: 10.4049/jimmunol.170.7.3866

http://www.jimmunol.org/content/170/7/3866

References

This article cites 62 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/170/7/3866.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Modulation of Cutaneous Inflammation by Angiotensin-Converting Enzyme

Thomas E. Scholzen,‡ Sonja Ständer,* Helge Riemann,* Thomas Brzoska,‡ and Thomas A. Luger*†

Cutaneous neurogenic inflammation is a complex biological response of the host immune system to noxious stimuli. Present evidence suggests that zinc metalloproteases may play an important role in the regulation of neurogenic inflammation by controlling the local availability of neuropeptides, such as substance P (SP), that are capable of initiating or amplifying cutaneous inflammation after release from sensory nerves. To address the hypothesis that the dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE) is capable of modulating skin inflammation, we have analyzed murine allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) using wild-type C57BL/6J (ACE+/+) or genetically engineered mice with a heterozygous deletion of somatic ACE (ACE+/−). In 2,4-dinitro-1-fluorobenzene-sensitized ACE+− mice, ACD was significantly augmented in comparison to ACE+/+ controls as determined by the degree of ear swelling after exposure to haptens. Likewise, systemic treatment of ACE−/− mice with the ACE inhibitor captopril before sensitization or elicitation of ACD significantly augmented the ACD response. In contrast, local damage and neuropeptide depletion of sensory nerves following capsaicin, injection of a bradykinin B2, or a SP receptor antagonist before sensitization significantly inhibited the augmented effector phase of ACD in mice with functionally absent ACE. However, in contrast to ACD, the response to the irritant croton oil was not significantly altered in ACE−/− compared with ACE+/+ mice. Thus, ACE by degrading bradykinin and SP significantly controls cutaneous inflammatory responses to allergens but not to irritants, which may explain the frequently observed exacerbation of inflammatory skin disease in patients under medication with ACE inhibitors. *The Journal of Immunology, 2003, 170: 3866–3873.

The biological functions of a number of neuropeptide mediators are terminated by proteolytic enzymes such as the zinc metalloproteases neutral endopeptidase (NEP; CD10) or angiotensin-converting enzyme (ACE) (1). The dipeptidyl carboxypeptidase ACE, also known as kininase II (EC 3.4.15.1; CD143), is a 150- to 180-kDa polypeptide chain containing two homologous extracellular domains bound to the cell membrane by a carboxyl-terminal hydrophobic anchor, each with an independent catalytic site. The main somatic ACE activity is associated with tissues such as the luminal side of the vascular endothelium or the renal epithelium. A soluble form of the enzyme that circulates in the plasma is also released after proteolytic cleavage of extracellular ACE (2–4). In addition to somatic ACE, a smaller testicular form of ACE also exists that is exclusively expressed by developing male germ cells (5) and appears to be of importance for male fertility (6). The main function of tissue ACE is the regulation of the local renin-angiotensin (Ang) system with the cleavage of Ang I into the vasoconstricting, aldosterone-releasing and blood pressure-rising peptide Ang II as most prominent conversion (7). In addition to NEP, ACE is the major proteolytic peptidase for the metabolism of kinins and tachykinins. ACE is capable of degrading the vasodilating and SP-inducing oligopeptide bradykinin (BK) to BK1–7 and further to the inactive molecule BK (1–5). Likewise, ACE degrades tachykinins such as substance P (SP) and other peptide mediators such as gastrin (1).

The neuropeptide SP is widely distributed in the central and peripheral nervous system, including the skin (8, 9). By activating neurokinin 1 receptors (NK-1R), SP is capable of inducing or augmenting a number of inflammatory responses including plasma extravasation, leukocyte activation, endothelial cell adhesion molecule expression, cytokine production, and mast cell activation (10–12). In the skin, neuropeptides such as SP are released from sensory nerves in response to noxious stimuli such as chemical, electrical, thermal, mechanical injury, and UV irradiation (13). In addition, BK is a powerful mediator capable of promoting symptoms of inflammation, including vasodilation, plasma extravasation, and pain by activating bradykinin receptors (B-R), in particular the B2-R (14, 15). Activation of B2-R on sensory nerves triggers the release of neuropeptides such as SP and calcitonin gene-related peptide, which results in the amplification of neurogenic inflammation (16).

The importance of ACE for the maintenance of blood pressure and renal function has been recently confirmed by the generation of mice with homozygous-targeted deletions of either somatic (tissue-bound and soluble) and testicular ACE (17) or by mice having only soluble ACE activity (6). In the ACE−/− animals, the lack of ACE resulted in a dramatic reduction of the blood pressure, indicating the absence of a mechanism that is capable of compensating for the loss of ACE. In addition, these mice had severely impaired
renal functions and the loss of testicular ACE resulted in a reduced fertility of male mice compared with wild-type animals (6, 17). Likewise, transient experimental NEP and ACE inhibition has been demonstrated to enhance the effects of exogenously applied or endogenously released kinins and tachykinins (18).

ACE inhibitors are widely used for the treatment of hypertension and myocardial infarction (19). However, a considerable number of patients experience adverse side effects such as dry cough and angioedema (20). In the skin, side effects include skin irritations, urticaria or angioedema, and exacerbation of inflammatory skin diseases such as psoriasis as well as of type I and type IV allergies (21). Little is known about a possible contribution of ACE to these inflammatory skin diseases. Therefore, in this study, we have tested the hypothesis whether the absence or short-term inhibition of ACE results in a dysregulated inflammatory skin response. Our results indicate that the acute or permanent inhibition of ACE results in an increased cutaneous inflammatory response to allergens but not to irritants. These results implicate the BK- and SP-proteolytic enzyme ACE as an important modulator of ACE inflammatory responses in the skin.

Materials and Methods

Animals

C57BL/6J mice were obtained from Harlan Winkelmann (Borchen, Germany). C57BL/6J-ACEtrans mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred as heterozygous animals. The ACEtrans targeted mutation knocks out only the somatic form of the ACE gene (22). Since homozygous mice showed a reduced postnatal viability, heterozygous animals (C57BL/6J-ACE+/−) were used in all experiments; wild-type littermates (C57BL/6J-ACE−/−) served as controls. Mice were housed in a barrier facility with free access to water and food according to local and state regulations. Males and females were studied at 10–14 wk with five to seven mice per experimental condition.

Experimentally induced allergic contact dermatitis (ACD) and administration of neuromodulating agents

ACD was induced as previously described (23). Mice (C57BL/6J-ACE−/− or wild-type littermate controls) were sensitized on day 0 by painting 25 µl of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB; Sigma-Aldrich, St. Louis, MO) in 4:1 acetone:olive oil on the shaved abdomen. Mice were challenged on day 5 by epicutaneous application of 10 µl of 0.2% DNFB in 4:1 acetone:olive oil on the dorsal surface of the right ear. The left ears were treated with vehicle alone (acetone:olive oil, 4:1) and served as an internal control for these studies. The ear swelling induced by the carrier alone was negligible. To detect any potential irritant effects of the hatchen, some mice were challenged with 10 µl of 0.2% DNFB on the right ear without previous sensitization. The ACD response was determined by the degree of ear swelling of the irritant-exposed ear compared with that of the vehicle-treated contralateral ear before irritant challenge and at 0–72 h after challenge as measured with a micrometer. In some experiments, the effect of B-R blockage on ACD was induced as previously described (24). The left ear was treated with vehicle only. The ACD response was determined by the degree of ear swelling of the irritant-exposed ear compared with that of the vehicle-treated contralateral ear before irritant challenge and at 0–24 h after challenge as measured with a micrometer. For some mice of each group ears were harvested after 6 h and processed for histological evaluation. Experiments were performed twice to verify reproducibility of the results.

Histology

Ears of three to four mice from each group were fixed in 10% PBS-buffered Formalin and 3–5-µm paraffin sections were stained with H&E and examined by light microscopy to assess histological changes and immune cell infiltration.

Electron microscopy

Biopsies from the abdominal skin of four mice treated with capsaicin or vehicle, respectively, were fixed in Karnovsky’s fixative, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epox. Ultrathin sections were prepared on an ultramicrotome (OMU6; Reichert). Ultrathin sections (70 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate. All of the ultrathin preparations were examined in a Philips CM10 electron microscope (Kassel, Germany).

Immunohistochemistry and immunofluorescence

Ears from three to four mice in each group were snap frozen, embedded in OCT compound (Miles, Elkhart, IN), cryostat sectioned, and fixed in acetonitrile for 20 min. Sections were processed for immunohistochemistry as described elsewhere (25). Slides were incubated with monoclonal rat anti-mouse CD4 or rat anti-mouse CD8 mAbs (BD Pharmingen, Heidelberg, Germany). Subsequently, slides were incubated with a secondary goat anti-rat Ab conjugated to peroxidase (BD Pharmingen, Heidelberg, Germany) and stained with 3-amin-9-ethylcarbazole substrate. Abs and irrelevant IgG (control) were diluted in PBS/1% BSA. For immunofluorescence technique, frozen sections (3–5 µm) of abdominal murine skin treated with capsaicin or vehicle, respectively, were preincubated for 30 min with 2% BSA. Specimens were then incubated overnight at 4°C with rabbit polyclonal Abs against SP (1:200; Genovis, Bad Homburg, Germany). After three 10-min washes in 0.1 M TBØ90.2% Triton X-100, sections were incubated with a porc FITC-labeled anti-rabbit Ab (1:50; DAKO, Hamburg, Germany). After final rinsing with TBS, sections were coveredslipped with Vectashield (Vector Laboratories, Burlingame, CA) and immediately investigated with a fluorescence microscope (Axioplan; Zeiss, Köln, Germany).

Statistical analysis

Results are expressed as mean ± SE. Differences between multiple groups were examined using ANOVA and Bonferroni’s test. Mean differences with p < 0.05 were considered to be significant.

Results

Enhanced ACD responses in mice after transient inhibition of ACE

To assess the role of ACE in modulating cutaneous inflammation, we compared the ACD response in normal mice (C57BL/6J) that in mice subjected to treatment with the ACE inhibitor captopril before sensitization. In DNFB-sensitized mice, application of DNFB to the right ear induced marked swelling in both untreated and captopril-treated animals (Fig. 1a). Notably, the response was significantly higher (50–65%) in mice subjected to captopril treatment before sensitization compared with mice not treated with the inhibitor. DNFB challenge in nonsensitized animals failed to induce a significant ear swelling in either the captopril-treated or

Downloaded from http://www.jimmunol.org/ by guest on November 13, 2017

The Journal of Immunology 3867

i.p., kindly provided by Dr. Emonds-Alt, Sanofi-Synthelabo, Montpellier, France). All studies were conducted three times to verify reproducibility of the results.

Induction of irritant contact dermatitis (ICD)

After measurement of the ear thickness of untreated animals, mice (C57BL/6J-ACE−/− and C57BL/6J-ACE+/− wild-type littermate controls, n = 10/group) were injected i.p. with 2.5 mg/kg captopril in 0.9% NaCl or 0.9% NaCl alone (control) 30 min before ICD induction by croton oil (0.8% in acetone; Sigma-Aldrich) as previously described (24). The left ear was treated with vehicle only. The ICD response was determined by the degree of ear swelling of the irritant-exposed ear compared with that of the vehicle-treated contralateral ear before irritant challenge and at 0–24 h after challenge as measured with a micrometer. For some mice of each group ears were harvested after 6 h and processed for histological evaluation. Experiments were performed twice to verify reproducibility of the results.
mice were treated with physiological concentrations of the specific kinin that this kinin is involved in augmented ACD responses, of neuropeptides such as SP from sensory nerves. To test the possibility that this kinin is involved in augmented ACD responses, mice were treated with physiological concentrations of the specific B2-R antagonist Hoe140. Intraperitoneal injection of Hoe140 before captopril treatment and DNFB sensitization significantly inhibited cutaneous inflammation 24, 48, and 72 h after challenge with DNFB (Fig. 1a). Likewise, depending on the time point analyzed, Hoe140 injection of mice without captopril also resulted in a reduction of the ACD ear swelling response by 31.5, 41.6, and 21.5% at 24, 48, and 72 h, respectively, after elicitation of ACD (p < 0.05 at 48 h, n = 5). These results indicate that the elevated ACD expression observed after inhibition of ACE during the sensitization phase also involves BK activation of B2-Rs. Repeated application of the neurotoxin capsaicin that activates vanilloid receptor 1 expressed on sensory c-fibers results in release and subsequent depletion of neuropeptides, including SP from sensory nerves (26). Therefore, we examined whether capsaicin is capable of inhibiting the enhancing effect of captopril on the ACD sensitization phase. An ointment containing 0.075% capsaicin was applied to the mouse abdominal skin twice daily over 5 days before systemic captopril treatment and DNFB sensitization. After Ag challenge, a profound reduction of the cutaneous inflammatory response in mice treated with capsaicin compared with animals not treated with this neurotoxin was observed (Fig. 1a). In animals treated with vehicle alone (ointment without capsaicin), no reduction in the augmented inflammatory response induced by captopril injection was detectable (data not shown). To elucidate the possibility that capsaicin treatment may interfere with DNFB sensitization involving a mechanism other than neuropeptide depletion, abdominal skin from mice treated multiple times with capsaicin was harvested and prepared for electron microscopy (Fig. 2). Examination of cutaneous nerves in these biopsies revealed that unmyelinated nerve fibers lose their intra-axonal structures, i.e., mitochondria and neurotrop-containing vesicles were destroyed in the skin of capsaicin-treated mice (Fig. 2b), but not in the skin of vehicle-treated control animals (Fig. 2a). Surrounding Schwann cells and large myelinated nerve fibers were unaffected. In addition, as determined by immunofluorescence, SP and calcitonin gene-related peptide were significantly less abundant when present in small subepidermal nerve fibers in the skin of mice subjected to capsaicin treatment (data not shown). Thus, capsaicin treatment of vehicle-treated mice, indicating that the reaction to DNFB was allergic and not due to an irritant effect of the DNFB solution (data not shown). Likewise, inhibition of ACE by captopril treatment of sensitized animals before DNFB challenge also significantly increased the ACD response compared with animals not treated with the ACE inhibitor (Fig. 1b). Thus, transient inhibition of ACE by captopril enhances hapten sensitization and expression of ACD.

Modulation of ACD response in captopril-treated mice by a B2-R antagonist or capsaicin

BK via the activation of B2-R is capable of triggering the release of neuropeptides such as SP from sensory nerves. To test the possibility that this kinin is involved in augmented ACD responses, mice were treated with physiological concentrations of the specific B2-R antagonist Hoe140. Intraperitoneal injection of Hoe140 before captopril treatment and DNFB sensitization significantly inhibited cutaneous inflammation 24, 48, and 72 h after challenge with DNFB (Fig. 1a). Likewise, depending on the time point analyzed, Hoe140 injection of mice without captopril also resulted in
murine skin damages cutaneous unmyelinated nerves and depletes them of SP and other neuropeptides which may impair sensitization, resulting in reduced ACD inflammatory responses.

The effect of ACE inhibition before sensitization on allergic skin inflammation was also investigated by histological examination of mouse ears after hapten challenge (Fig. 3). No significant differences were observed in the skin of sensitized animals treated with vehicle only (Fig. 3, a and b). Likewise, no differences were observed between DNFB-challenged ears of nonsensitized, untreated, or captopril-treated mice (data not shown). However, in sensitized, captopril-treated animals, severe dermal edema, an intense inflammatory infiltration consisting predominantly of neutrophils and mononuclear cells, and some epidermal hyperplasia were detected 72 h after Ag challenge (Fig. 3c). Notably, edema, cellular infiltrate, and epidermal hyperplasia were clearly less prominent in sensitized but not captopril-treated C57BL/6J mice (Fig. 3d). Treatment of mice with either capsaicin or the B2-R antagonist before DNFB sensitization resulted in a reduction of cutaneous edema and leukocyte infiltration (data not shown). In addition, staining of ear tissue with Abs against CD4 or CD8 (Fig. 4) revealed a clear increase of CD4+ and CD8+ cells in the ears of sensitized and challenged C57BL/6J mice (Fig. 4e and f) in comparison to control ears of mice not subjected to ACD elicitation (Fig. 4b and c). Importantly, in ears from captopril-treated, sensitized, and challenged mice a higher density of CD4+ and CD8+ T cells could be detected 72 h after Ag challenge (Fig. 4h and i) in comparison to animals not treated with the ACE inhibitor before sensitization (Fig. 4e and f). Moreover, capsaicin treatment...
before ACE inhibition and sensitization resulted in a reduction in the density of both CD4+ and CD8+ cells (Fig. 4, k and l). These results indicate that ACE inhibition before sensitization results in an enhanced recruitment of CD4+CD8+ T cells after Ag challenge during the effector phase of ACD.

**ACD responses in C57BL/6J-ACE−/− and C57BL/6J-ACE+/+ mice**

To verify that the effects of captopril on ACD are specific for ACE and not due to the inhibition of other peptidases, the ACD response in mice with a targeted deletion of the somatic form of ACE was examined. Due to a reduced postnatal viability of homozygous animals, heterozygous mice were used in these experiments (ACE−/−; Fig. 5). When C57BL/6J-ACE−/− were sensitized and challenged with DNFB, a significant increase of the allergic ear swelling response in comparison to sensitized and challenged wild-type animals 24 h after Ag challenge was observed (Fig. 5a). Importantly, this increased ACD response could be significantly reduced by injection of a highly specific nonpeptide NK-1R antagonist (SR140333) before sensitization (Fig. 5b). These data indicate that temporary inhibition of ACE before sensitization or challenge as well as partial genomic deletion of ACE results in significantly increased ACD inflammatory response in mice, which require sensory nerves and involve BK-mediated B2-R and SP-mediated NK-1R activation.

**Similar ICD responses in ACE−/− and ACE+/+ mice**

When ACE−/− and ACE+/+ mice were treated with the topically applied irritant croton oil, the ear swelling response to the irritant was time-dependent increased in ACE+/+ and wild-type animals, reaching a peak swelling at ~6 h after croton oil challenge (Fig. 6). However, in contrast to the results observed during ACD, the ICD response to croton oil in mice bearing a partial ACE deletion was not significantly different from that in wild-type ACE+/+ animals (Fig. 6). Upon histological examination, the cellular infiltration as well as the tissue morphology in ICD was also not visibly different in ACE+/+ mice compared with ACE+/+ (data not shown). Thus, ACE is an important inflammatory modulator of ACD responses, but not of ICD cutaneous inflammation.

**Discussion**

The local tissue availability and bioactivity of BK as well as those of SP appear to be tightly controlled by their release from cells and sensory nerves, the presence of B-R or NK-1R on target cells, and local expression of kinin/tachykinin-degrading enzymes such as NEP or ACE (1, 18, 27, 28). The role of NEP in modulating neuropeptide-mediated biological responses and systemic inflammation is supported by a number of recent in vivo studies demonstrating that NEP−/− animals are highly sensitive to bacterial endotoxins (29) and the absence of NEP promotes intestinal (30) as well as cutaneous inflammatory response to allergens (24). Likewise, transient inhibition of ACE was shown to promote neurogenic inflammation in mice, i.e., plasma extravasation, vasodilation (31, 32), and experimental dermatitis (33–35). Accordingly, ACE inhibition enhanced short-term (<24 h) skin inflammation induced by OVA, capsaicin, or BK injection although the extent of inflammation was dependent on the ACE inhibitor used (34, 35).

In the present study, we have used murine models for allergic and ICD to study the relative contribution of ACE in cutaneous inflammation. Our results provide substantial evidence for a major role for ACE in ACD. Inflammatory responses to the hapten DNFB over 72 h were markedly greater in sensitized animals partially lacking ACE or in wild-type mice treated with physiological concentrations of an ACE inhibitor. This supports the hypothesis that ACE contributes to the down-regulation of neuroinflammatory responses. Importantly, in our study, the use of animals partially lacking ACE demonstrated that increased ACD inflammatory responses are truly due to the inactivation of ACE and not due to a
drug-dependent nonspecific inhibition of other proteolytic peptides. This is in accordance with recent studies demonstrating that even heterozygous mice, which only partially lacked somatic and testicular ACE, displayed significantly decreased ACE activity (22) and blood pressure responses to exogenous ACE substrates such as Ang I and BK (36). In our study, the enhanced expression of ACD observed in mice after temporary inhibition or heterozygous deletion of ACE was prevented by the local depletion of SP from cutaneous sensory nerves at the site of sensitization or by administration of B2-R or NK-1R antagonists before sensitization. We have previously demonstrated that neuropeptide depletion from sensory nerves by repeated treatment with the neurotoxin capsacain profoundly reduces the efferent ACD response in NEP-deficient mice (24). Likewise, another study demonstrated that inhibition of ACE in mice increased plasma extravasation involving BK as an important mediator of neurogenic inflammation (31). In extension of these data, our observations support the role of an intact functional peripheral sensory nervous system in ACD sensitization and demonstrates that impaired degradation of BK and also of SP during ACD sensitization contributes to allergic inflammatory effector responses in the skin. In contrast, ICD responses appear to be less affected by the functional absence of ACE. Although initial ICD ear swelling responses in ACE−/− mice were slightly increased compared with those of wild-type animals, this difference was not significant. This confirms our previous data using NEP−/− mice demonstrating that the lack of NEP does not affect ICD responses to croton oil or SDS (24). However, other studies also demonstrated an involvement of SP and sensory nerves in both allergic and irritant inflammation (37–41). The precise mechanism of this difference between nonspecific irritant inflammation and the hapten-specific ACD response in mice with functionally inactive proteolytic peptides such as ACE or NEP compared with wild-type animals is not entirely clear. ACD responses, in contrast to ICD, require contact between APC; i.e., Ag-loaded dendritic cells (DC) and T cells in the T cell zones of secondary lymphoid organs such as draining lymph node as well as the expression of the IL-2Rα-chain (CD25) (57). In addition, the absence of DC, proliferation of T cells induced by direct CD3/CD28 ligation was partly dependent on signaling through NK1-R, revealing an autocrine effect of SP production by T cells (46). Importantly, T cells have been demonstrated to express ACE and in vitro captopril treatment of T cells, resulting in reduced ACE activity (60), promoted T cell proliferation induced by ConA (61). Thus, blocking of SP degradation by transient inhibition of ACE might result in the local or systemic generation of a distinct cytokine environment involving IL-12 and IFN-γ that may be capable of promoting a Th2-mediated cellular immune response. However, the precise mechanism of the increased ACD inflammatory response triggered by ACE inhibition before elicitation of ACD is not clear. As demonstrated previously, elicitation of ACD can be dissection into an hapten-specific effector cell activation and a relatively unspecific proinflammatory signal, which is due to the fact that hapten acts also as irritants (62–65). In this scenario, impaired neuropeptide degradation might result in both activation of Ag-specific T cells as well as the generation of an unspecific proinflammatory skin environment at the site of Ag challenge that conditions the tissue for a full Ag-specific ACD elicitation.

In summary, our data indicate that the absence or reduced activity of ACE during sensitization leads to the development of augmented responses to allergens in a murine ACD model system which may be due to the prolonged tissue presence of released SP and BK. Our observations further support the hypothesis that the expression and regulation of proteolytic peptides such as ACE or NEP and of SP and NK-1R play an important role in inflammatory skin diseases such as ACD. This could be of importance for the clinical use of ACE and possibly NEP inhibitors, since application of these drugs may, under certain circumstances and predispositions, promote the induction of type IV hypersensitivity reactions. In addition, it appears to be conceivable that ACE inhibition may trigger expression of allergic inflammatory responses in already sensitized individuals. These findings further suggest the notion that in addition to cytokines, neuropeptides play an important role in the regulation of immune and inflammatory responses.
Acknowledgments

We thank Michaela Fastrich, Karin Fischer, and Andrea Wissel for their expert technical assistance. The NK-1R antagonist SR140333 was kindly provided by Dr. Emonds-Alt (Sanofi-Synthélabo, Montpellier, France). We are grateful to Stephanie Grabbe for carefully reading this manuscript.

References