Chapter 10

Age-induced airway hyperresponsiveness in guinea pigs due to increased arginase activity and peroxynitrite

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Summary
Recently, an association between age and increased airway responsiveness has been reported. In the vasculature, aging - characterized by increased vascular smooth muscle contractility - is associated with reduced endothelial nitric oxide (NO) production and increased generation of peroxynitrite, the reaction product of NO and superoxide anion. Arginase may promote peroxynitrite production by attenuating L-arginine availability to NO-synthase (NOS), which favors the synthesis of both NO and superoxide anion by this enzyme. In the present study, we studied the roles of NO, superoxide anion and peroxynitrite as well as of arginase in age-related changes of airway responsiveness. To this aim, airway responsiveness to methacholine was measured in perfused tracheal preparations of young (3-5 months), mature (7-11 months) and senescent (20-23 months) guinea pigs. In tracheae from mature and senescent animals, methacholine-induced airway constriction was increased (1.73 and 1.94-fold, respectively) when compared to young adults. Incubation with the NOS inhibitor L-NAME as well as with the superoxide anion scavenger SOD reduced this airway hyperresponsiveness in both aged groups to the level of young controls, indicating that NO and superoxide anion are involved, presumably via the formation of peroxynitrite. Interestingly, airway hyperresponsiveness in mature guinea pigs was also normalized by exogenous L-arginine as well as after inhibition of arginase using the specific inhibitor nor-NOHA, which was reversed by L-NAME. Moreover, arginase activity in tracheal homogenates of mature animals is markedly increased. In conclusion, airway reactivity is increased in aging guinea pigs due to the formation of peroxynitrite, which may be promoted by increased arginase activity by attenuating the availability of L-arginine to NOS.

Introduction
Nitric oxide (NO) is importantly involved in the regulation of airway function and is produced by NO synthase (NOS), which utilizes the semi-essential amino acid L-arginine, oxygen and NADPH to produce NO and L-citrulline [1]. NO induces bronchodilation by causing airway smooth muscle relaxation in a cyclic GMP (cGMP) dependent as well as independent way [2]. Up to now, three NOS isoenzymes have been identified. Neuronal (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) are constitutively expressed in the airways, mainly in inhibitory nonadrenergic noncholinergic neurons (nNOS), endothelium (eNOS) and epithelium (nNOS and eNOS) [2]. Inducible NOS (iNOS or NOS II), induced by proinflammatory cytokines during airway inflammation, is mainly expressed in macrophages and epithelial cells [2]. The substrate availability to NOS is an important determinant of the production of NO in the airways and is regulated by another L-arginine metabolizing enzyme, arginase, yielding L-ornithine and urea [3-6][Chapters 6&7]. Using intact guinea pig tracheal preparations, we have demonstrated that
endogenous arginase attenuates the production of agonist-induced epithelial as well as neuronal constitutive NOS (cNOS)-derived NO by limiting substrate availability to NOS [5,6][Chapter 6].

In the vasculature, it has been demonstrated that aging is associated with the formation of procontractile peroxynitrite, the reaction product of NO and superoxide anion [7]. While both eNOS expression and activity are upregulated, vascular levels of NO are markedly reduced in aged as compared to young rats [7]. In addition, both the production of superoxide anions and staining for 3-nitrotyrosine, a marker for peroxynitrite, are increased in aged animals [7]. Taken together, these findings indicate that scavenging of NO by superoxide anions, leading to increased generation of procontractile peroxynitrite, is involved in reduced endothelium-dependent vascular smooth muscle relaxation with aging [7]. Furthermore, increased levels of nitrotyrosilated mitochondrial manganese superoxide dismutase were found, leading to decreased endogenous superoxide anion scavenging capacity [7]. A role for superoxide anions in reducing the bioavailability of NO and subsequent formation of peroxynitrite has also been indicated in age-related increase of electrical field stimulation-induced contractions of mesenteric arteries from spontaneous hypertensive rats [8].

In the airways, peroxynitrite is importantly involved in the development of airway hyperresponsiveness after the allergen-induced late asthmatic reaction (LAR). Thus, in perfused guinea pig tracheal preparations from animals after the LAR we demonstrated that both the nonselective NOS inhibitor N\textsuperscript{\omega}-nitro-L-arginine methyl ester (L-NAME) as well as the superoxide anion scavenger superoxide dismutase (SOD) strongly decreased the airway hyperresponsiveness after the late asthmatic reaction, indicating that both NO and superoxide anion are involved, presumably via the formation of peroxynitrite [9]. Recently, we reported that increased L-arginine utilization by upregulated arginase activity in the airways underlies the formation of peroxynitrite after the LAR, by limiting the L-arginine availability to iNOS [10]. Indeed, it has been demonstrated in macrophages that, under conditions of low L-arginine, iNOS produces both NO, through the oxygenase moiety of the enzyme, and superoxide anions, through its reductase moiety, leading to a highly efficient synthesis of peroxynitrite [11,12]. Interestingly, a role for arginase in age-induced alterations of vascular function has also been suggested [13,14]. Thus, arginase activity is increased with aging in rat aortic rings, leading to reduced NOS activity [13]. In addition, while arginase activity is increased in rabbit cavernous tissue of aged animals, tissue content of L-arginine and cGMP production are markedly decreased, causing reduced vascular smooth muscle relaxation [14].

Interestingly, a recent analysis by Scichilone and coworkers showed a positive relationship between age and increased airway responsiveness [15]. However, the mechanism underlying this age-related change in airway responsiveness is currently unknown. In the present study, we investigated the effect of age on airway responsiveness in young adult (3-5 months), mature (7-11 months) and senescent
(20-23 months) guinea pigs. This was assessed both in vivo, by inhalation of histamine, and ex vivo, using perfused tracheal preparations. The roles of NO, superoxide and peroxynitrite in age-related changes in airway responsiveness were assessed using L-NAME and SOD in perfused tracheal preparations of all age groups. The role of L-arginine availability and of arginase in airway responsiveness was studied using exogenous L-arginine and the specific arginase inhibitor Nω-hydroxy-nor-L-arginine (nor-NOHA) in these preparations. In addition, arginase activity was measured in tracheal homogenates of young adult and mature animals.

**Methods**

**Animals**

Outbred male specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, UK) were used in this study. The animals were group-housed in individual cages in climate-controlled animal quarters and given water and food ad libitum, while a 12-h on/12-h off light cycle was maintained. Animals of three different age groups were used, 3-5 months (young adults), 7-11 months (mature) and 20-23 months (senescent). All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

**Tracheal perfusion**

Tracheal perfusion experiments were performed as previously described [16]. The tracheae were rapidly removed and placed in Krebs-Henseleit solution (37°C) of the following composition (mM): NaCl 117.50, KCl 5.60, MgSO4 1.18, CaCl2 2.50, NaH2PO4 1.28, NaHCO3 25.00, D-glucose 5.50; gassed with 5% CO2 and 95% O2; pH 7.4. The tracheae were prepared free of serosal connective tissue and cut into two halves of approximately 17 mm before mounting in a perfusion setup. To this aim, the tracheal preparations were attached at each end to stainless steel perfusion tubes fixed in a Delrin perfusion holder. The holder with the trachea was then placed in a water-jacketed organ bath (37°C) containing 20 ml of gassed Krebs-Henseleit (the serosal or extraluminal (EL) compartment). The lumen was perfused with recirculating Krebs-Henseleit from a separate 20 ml bath (mucosal or intraluminal (IL) compartment) at a constant flow rate of 12 ml/min. Two axially centred side-hole catheters connected with pressure transducers (TC-XX,Viggo-Spectramed B.V., Bilthoven, The Netherlands) were situated at the distal and proximal ends of the trachealis to measure hydrostatic pressures (P_{outlet} and P_{inlet}, respectively). The signals were fed into a differential amplifier to obtain the difference between the two pressures ($\Delta P = P_{inlet} - P_{outlet}$), which was plotted on a flatbed chart recorder (BD 41, Kipp en Zonen, Delft, The Netherlands). $\Delta P$ reflects the resistance of the tracheal segment to perfusion and is a function of the mean diameter of the trachea between the pressure taps.

After a 45 minutes equilibration period with three washes with fresh KH (both IL and EL), 1 μM (-)-isoproterenol was added to the EL compartment for maximal
smooth muscle relaxation to assess basal tone. After three washes during at least 30 minutes, the trachea was exposed to 40 mM KCl (EL) to obtain a receptor-independent reference response. Subsequently, the preparation was washed 4 times with KH during 45 minutes until basal tone was reached and a cumulative concentration response curve was made with IL administered methacholine. When used, L-NAME (0.1 mM) and nor-NOHA (5.0 µM) were applied to the IL reservoir [5,17], while superoxide dismutase (SOD, 100 U/ml) and L-arginine (5.0 mM) were applied to the EL reservoir, all 40 min prior to agonist-addition [3,9].

**Measurement of airway responsiveness in vivo**

Histamine provocations were performed by inhalation of aerosolized solutions. These provocations were carried out in a specially designed perspex cage of 9 L, in which the guinea pigs could move freely. A DeVilbiss nebulizer (type 646) driven by an airflow of 8L/min provided the aerosol with an output of 0.33 ml/min. The animals were habituated to the experimental conditions and the provocations procedures, as described previously [18,19].

In order to assess airway reactivity for histamine, provocations with increasing dosage steps of 25 µg/ml solution in saline were performed. Histamine provocations lasted 3 min and were separated by 7 min intervals. Animals were challenged until the first signs of respiratory distress were observed which lasted for at least 5 min. The nebulized histamine dose to induce this respiratory distress is called the histamine threshold. Histamine threshold-values were assessed twice, with an one week interval.

**Arginase assay**

Arginase activity in the tracheal homogenates from young adult and mature guinea pigs was determined by measuring the conversion of L-[guanido-14C]arginine to [14C]urea as previously described [17,20][Chapter 2]. The specificity of the assay in measuring arginase activity was confirmed by the inhibitory effect of nor-NOHA [17][Chapter 2]. Protein concentrations were determined by the Bradford Coomassie Brilliant Blue method [21] using bovine serum albumin as a standard.

**Data analysis**

IL responses of the tracheal tube preparations to methacholine were expressed as a percentage of the response induced by EL administration of 40 mM KCl to correct for differences in baseline ∆P and in ∆P changes in response to contractile stimuli due to variation in internal diameter of the preparations used. The contractile effect of 10 mM methacholine (highest concentration) was defined as E\text{max} [16]. Using this E\text{max}, the sensitivity to methacholine was evaluated as pEC\text{50}. In vivo airway responsiveness was expressed as the histamine dose causing respiratory distress (i.e. the histamine threshold). Arginase activity was expressed as pmol urea produced per mg protein per minute.
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The results are expressed as means ± SEM. Statistical analysis was performed using the Student's t-test for unpaired observations. Differences were considered statistically significant at \( P<0.05 \).

**Chemicals**

(-)-isoprenaline hydrochloride, \( \text{N}^{\omega} \)-nitro-L-arginine methyl ester, L-arginine hydrochloride, superoxide dismutase and histamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and methacholine chloride from Aldrich (Milwaukee, WI, USA). L-[guanido-\( ^{14} \text{C} \)]arginine (specific activity 51.5 mCi/mmol) was obtained from New England Nuclear Life Science Products, Inc (Boston, MA, U.S.A.). \( \text{N}^{\omega} \)-hydroxy-nor-L-arginine was kindly provided by Dr J.-L. Boucher (Université Paris V, France).

**Results**

Methacholine-induced constriction of perfused guinea pig airways was significantly increased in both aging groups compared to the young adults. A 1.73-fold and 1.94-fold \( (P<0.005 \text{ both}) \) increase of the \( E_{\text{max}} \) to methacholine was observed in the airways of mature and senescent guinea pigs, respectively (Figure 1, Table 1), without an effect on the sensitivity (pEC\(_{50}\)) to the agonist.

![Figure 1: Methacholine-induced constriction of intact perfused tracheae from young controls (3-5 months), mature (7-11 months) and senescent (20-23 months) guinea pigs. Results are means ± SEM of 3-12 experiments.](image-url)
We also determined airway responsiveness in vivo by determining airway responsiveness to inhaled histamine. Interestingly, in vivo airway responsiveness of the mature guinea pigs, as determined by histamine threshold-values, was significantly increased ($P<0.001$) compared to young adults (Figure 2). In line with the results obtained using isolated perfused airways, the in vivo airway responsiveness was also increased in senescent guinea pigs ($P<0.01$; Figure 2), to a similar extent as the mature animals.

**Figure 2:** Airway responsiveness in vivo in young adult, mature and senescent guinea pigs as expressed by histamine threshold-values. Results are means ± SEM of 3-7 experiments. **$P<0.01$ and ***$P<0.001$ compared to young adults.

In line with previous observations [3,5,9,16,17][Chapter 2], incubation of the isolated perfused tracheae with the non-specific NOS-inhibitor L-NAME (0.1 mM, IL) significantly increased the responsiveness to methacholine in the young adult animals, while incubation with SOD slightly decreased airway responsiveness (Figure 3A, Table 1). The increased airway responsiveness induced by L-NAME in the young adults was similar to the airway hyperresponsiveness observed in the aging guinea pigs of both age-groups. Interestingly, the observed airway hyperresponsiveness in both aged groups was markedly reduced after inhibiting NOS activity. Thus, incubation with L-NAME (0.1 mM, IL) caused a significant decrease in airway responsiveness in the mature guinea pigs as well as in the senescent animals by 82.5% ($P<0.05$) and 83.8% ($P<0.001$), respectively, without significant effects on the pEC$_{50}$-values (Figure 3B and C; Table 1). The hyperresponsiveness in both the mature and senescent guinea pigs was similarly reduced by the superoxide anion scavenger SOD ($P<0.05$), again without significantly affecting the sensitivity to the agonist (Figure 3B and C, Table 1).
Incubation with exogenous L-arginine (5.0 mM) also significantly reduced airway hyperresponsiveness in the mature guinea pigs by 50.6% ($P<0.05$, Figure 4, Table 1), indicating that limitation of L-arginine underlies the development of hyperresponsiveness with aging. Although the hyperresponsiveness is reduced after administration of L-arginine, responsiveness is still 1.36 fold increased as compared to young adults ($P<0.05$; Figure 4, Table 1). Interestingly, the hyperresponsiveness in the mature guinea pigs was completely reduced to the level of young adults by the arginase inhibitor nor-NOHA (5.0 mM; $P<0.05$), without affecting the sensitivity (Figure 5, Table 1). The effect of nor-NOHA on $E_{\text{max}}$ could be fully reversed after coincubation with L-NAME (1.0 mM; $P<0.05$), to the hyperreactive level of untreated airways from mature animals (Figure 5, Table 1)
We also determined arginase activity in tracheal homogenates from young and mature guinea pigs. Tracheal arginase activity of the mature guinea pigs was more than 3-fold increased as compared to young controls ($P<0.05$, Figure 6).
Figure 6: Arginase activity measured in tracheal homogenates of young adult and mature guinea pigs. Arginase activity, expressed as pmol urea produced per mg protein per minute, was measured for 20 minutes in the presence of 1.66 mM L-arginine. Results are means ± SEM of 4-5 experiments. *P<0.05 compared to young controls.

Table 1: Effects of SOD, L-NAME, nor-NOHA and/or L-arginine on the responsiveness to methacholine of intact perfused tracheae from young adult and aging guinea pigs.

<table>
<thead>
<tr>
<th></th>
<th>E_{max} ( % KCl )</th>
<th>pEC_{50} (-log M )</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young adult</td>
<td>50.6±2.5</td>
<td>2.91±0.12</td>
<td>8</td>
</tr>
<tr>
<td>0.1 mM L-NAME</td>
<td>95.6±7.3**</td>
<td>3.22±0.14</td>
<td>5</td>
</tr>
<tr>
<td>100 U/ml SOD</td>
<td>36.0±4.5**</td>
<td>2.73±0.06</td>
<td>7</td>
</tr>
<tr>
<td>Mature</td>
<td>87.6±4.9***</td>
<td>3.14±0.12</td>
<td>12</td>
</tr>
<tr>
<td>0.1 mM L-NAME</td>
<td>57.3±4.4****</td>
<td>3.07±0.09</td>
<td>15</td>
</tr>
<tr>
<td>100 U/ml SOD</td>
<td>40.4±6.8****</td>
<td>3.32±0.23</td>
<td>6</td>
</tr>
<tr>
<td>5.0µM nor-NOHA</td>
<td>55.8±8.9***</td>
<td>3.16±0.15</td>
<td>6</td>
</tr>
<tr>
<td>+ 1.0 mM L-NAME</td>
<td>87.2±10.6†</td>
<td>2.77±0.24</td>
<td>4</td>
</tr>
<tr>
<td>5.0 mM L-arginine</td>
<td>68.8±7.5**†</td>
<td>3.08±0.14</td>
<td>6</td>
</tr>
<tr>
<td>Senescent</td>
<td>97.8±1.8***</td>
<td>3.20±0.07</td>
<td>3</td>
</tr>
<tr>
<td>0.1 mM L-NAME</td>
<td>58.2±4.5§§</td>
<td>3.01±0.15</td>
<td>4</td>
</tr>
<tr>
<td>100 U/ml SOD</td>
<td>41.2±11.0§§</td>
<td>3.20±0.14</td>
<td>4</td>
</tr>
</tbody>
</table>

Results are means ± SEM of n experiments. **P<0.01 and ***P<0.001 compared with the untreated young adult group; *P<0.05, **P<0.01 and ***P<0.005 compared with the untreated mature group; †P<0.05 compared with nor-NOHA treated; §P<0.01 and §§P<0.001 compared with the untreated senescent group.
Discussion

Methacholine-induced constriction of perfused tracheal preparations ex vivo was significantly increased in both mature and senescent guinea pigs as compared to young adults. The importance of this finding is illustrated by the observation that in vivo airway responsiveness to inhaled histamine was increased as well. Interestingly, as reviewed by Scichilone and coworkers, a positive relationship between age and increased airway responsiveness is also found in humans [15]. In the present study, increased formation of procontractile peroxynitrite appeared to be involved in the age-related airway hyperresponsiveness of both mature and senescent animals, since the NOS inhibitor L-NAME and the superoxide anion scavenger SOD decreased airway hyperresponsiveness to a similar extent. The observed changes are strikingly similar to the results observed in guinea pig airways obtained after the allergen-induced late asthmatic reaction, in which the airway hyperresponsiveness – induced by inhalation of an allergen – was also inhibited by L-NAME and SOD [9].

Increased formation of peroxynitrite from NO and superoxide, and age-induced changes in physiological responses have previously been observed in the vasculature. Thus, despite an increase in eNOS expression and activity, levels of NO are markedly reduced in aorta’s of aged rats, while the production of superoxide anions as well as staining for 3-nitrotyrosine, a marker for peroxynitrite, are increased [7]. Furthermore, increased levels of nitrotyrosilated mitochondrial manganese SOD were detected, leading to a decreased superoxide anion scavenging capacity and hence increased formation of peroxynitrite [7]. It was also observed that, unlike the expression of endogenous SOD, the activity of the enzyme is significantly decreased in hearts from aged versus young rats [22]. These findings indicate that reduced endogenous superoxide anion scavenging capacity with aging leads to an increased reaction of superoxide anions with NO. This causes an increased formation of procontractile peroxynitrite and reduced levels of NO, which both contribute to a reduced endothelium-dependent vascular smooth muscle relaxation with aging [7,22]. Moreover, an age-related increase in electrical field stimulation-induced contractions of mesenteric arteries of spontaneous hypertensive rats due to reduced NO bioavailability has been reported [8]. Although electrical field stimulation-induced nNOS-derived NO production was increased with aging, increased production of superoxide anion caused a reduced bioavailability of NO and increased peroxynitrite formation [8].

A major role for peroxynitrite in the development of airway hyperresponsiveness was previously found after the allergen-induced late asthmatic reaction, when L-NAME as well as SOD decreased the airway hyperresponsiveness, indicating that both NO and superoxide anion are involved [9]. Recently, we demonstrated that increased arginase activity in the airways is strongly involved in the formation of peroxynitrite after the LAR by limiting the L-arginine availability to iNOS [10]. In
asthmatics, increased nitrotyrosine staining, as a marker for peroxynitrite, was observed in the airway epithelium and inflammatory cells, which correlated with iNOS expression, airway hyperresponsiveness and inflammation [23]. Peroxynitrite is procontractile and proinflammatory [9,23,24] and is known to induce degranulation of eosinophils, epithelial damage and AHR [25] as well as airway microvascular hyperpermeability [26] in guinea pigs. Remarkably, the airway hyperresponsiveness to methacholine in preparations from mature guinea pigs was reduced by exogenous L-arginine, indicating that limitation of substrate to NOS underlies the observed hyperresponsiveness in the mature animals. One mechanism which may lead to a reduced availability of substrate to NOS is increased L-arginine utilization by arginase. Although classically considered to be an enzyme of the urea cycle in the liver, arginase is also expressed in extrahepatic tissues, including the lung [27,28], where it regulates NO synthesis by limiting the availability of intracellular L-arginine for NOS [27-30]. Two distinct isoforms of arginase have been identified in mammals: the cytosolic arginase I, mainly expressed in the liver, and the mitochondrial arginase II, which is mainly expressed in extrahepatic tissues [28]. Both arginase isoforms are constitutively expressed in the airways, particularly in the bronchial epithelium and in fibroblasts from peribronchial connective tissue [27]. We have previously demonstrated that endogenous arginase activity is functionally involved in the regulation of airway smooth muscle tone by attenuating both neuronal and agonist-induced nonneuronal NO production [5,6]. It was also found that increased arginase activity after the allergen-induced early asthmatic reaction importantly contributes to the deficiency of both neuronal and nonneuronal NO [4,16,17][Chapters 2&7]. The importance of increased arginase in the pathophysiology of allergic asthma was recently supported in mouse models [31,32] and in asthmatic patients [31,33]. In addition, arginase activity is increased by the Th2-cytokines IL-4 and IL-13 [31,34]. In the present study we demonstrated that inhibition of arginase by the specific inhibitor nor-NOHA completely reversed the airway hyperresponsiveness to methacholine in the mature animals to the responsiveness observed in young adults. Moreover, arginase activity in tracheal homogenates of mature guinea pigs was markedly increased as compared to young adults, suggesting that increased arginase activity underlies the L-arginine limitation and airway hyperresponsiveness in aging guinea pigs. Since coincubation with L-NAME reversed the normalizing effect of nor-NOHA, it can be concluded that arginase inhibition reduces the airway responsiveness by restoring the production of bronchodilating NO.

It has been established that at low L-arginine concentrations all three NOS isoforms can produce both NO and superoxide, leading to the rapid formation of peroxynitrite [12,35,36]. While the NO production by NOS is stimulated when increasing the substrate availability, the superoxide anion production is reduced [37]. Therefore, we postulate that increased arginase activity in aging contributes to a
limitation of L-arginine to NOS, leading to the production of both NO and superoxide anion by NOS and subsequent formation of the procontractile peroxynitrite. Inhibition of arginase increases the availability of L-arginine to NOS, thereby reducing the production of superoxide anions and hence peroxynitrite, and restoring the production of free bronchodilating NO.

In line with our present findings in the airways, increased arginase has recently been observed in vascular aging. In aortic preparations from aged rats, increased arginase activity contributes to endothelial dysfunction by reducing the NOS activity and cGMP levels [13,38]. In addition, arginase I mRNA and protein as well as arginase II mRNA, but not protein expression, were increased with aging in rat aorta, with arginase I being the predominant isoform [38]. Inhibition of arginase activity as well as knockdown of arginase I restored NOS activity, cGMP and NO levels as well as endothelial function [13,38]. In aging rabbits, increased arginase activity contributes to impaired NO-mediated cavernous relaxations by limiting the L-arginine availability to NOS [14]. Thus, impaired relaxations in aged rabbits could also be normalized by arginase inhibition as well as with exogenous L-arginine [14]. Therefore, it can be concluded that increased arginase activity importantly contributes to the age-related changes in both the vascular and respiratory system by limiting the availability of L-arginine to NOS.

In conclusion, in mature and senescent guinea pigs, airway responsiveness to methacholine is significantly enhanced due to increased formation of peroxynitrite. This is caused by increased arginase activity, which attenuates L-arginine availability to NOS, leading to simultaneous production of NO and superoxide anion by this enzyme.

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