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REVIEW

An imbalance in C/EBPs and increased mitochondrial activity in asthmatic airway smooth muscle cells: novel targets in asthma therapy?

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The asthma prevalence was increasing over the past two decades worldwide. Allergic asthma, caused by inhaled allergens of different origin or by food, is mediated by inflammatory mechanisms. The action of non-allergic asthma, induced by cold air, humidity, temperature or exercise, is not well understood. Asthma affects up to 15% of the population and is treated with anti-inflammatory and muscle relaxing drugs which allow symptom control. Asthma was first defined as a malfunction of the airway smooth muscle, later as an imbalanced immune response of the lung. Recent studies placed the airway smooth muscle again into the focus. Here we summarize the molecular biological basis of the deregulated function of the human airway smooth muscle cell as a cause or important contributor to the pathology of asthma. In the asthmatic human airway smooth muscle cells, there is: (i) a deregulation of cell differentiation due to low levels of maturation-regulating transcription factors such as CCAAT/enhancer binding proteins and peroxisome proliferator-activated receptors, thereby reducing the cells threshold to proliferate and to secrete pro-inflammatory cytokines under certain conditions; (ii) a higher basal energy turnover that is due to increased number and activity of mitochondria; and (iii) a modified feedback mechanism between cells and the extracellular matrix they are embedded in. All these cellular pathologies are linked to each other and to the innate immune response of the lung, but the sequence of events is unclear and needs further investigation. However, these findings may present the basis for the development of novel curative asthma drugs.
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Abbreviations: C/EBP, CCAAT/enhancer binding protein; DNA, deoxyribonucleic acid; eIF-4E, eukaryotic initiation factor-4E; IgE, immunoglobulin E; IL, interleukin; PPAR, peroxisome proliferator-activated receptor; TGF, tumour growth factor

Asthma is a chronic inflammatory disease of the lung which affects 8–15% of the population. The prevalence of asthma was increasing over the past two decades, but recent evidence suggests that it is slowing down, at least in western countries. The reason for the increase in asthma remains unknown and most studies have related this phenomenon to changes of lifestyle or to changes in the environment (Devenny *et al.*, 2004; Masoli *et al.*, 2004; Lee *et al.*, 2005; Eder *et al.*, 2006; Partridge, 2007). Asthma attacks can be associated with inhaled allergens such as grass pollens, animal hair, house dust mite's faeces or by food (Kay, 2001a,b; Devenny *et al.*, 2004; London, 2007). However, there are other non-allergic triggers of asthma such as inhalation of cold air, sudden changes in air humidity or temperature, and related to these causes is exercise induced asthma (Stensrud *et al.*, 2006; Knöpfli *et al.*, 2007; Koskela, 2007; London, 2007). Asthma can occur from birth or can start at any stage of life; again the reasons for these variations are largely unknown (Atwood and Bowen, 2008; Litonjua and Gold, 2008; Panettieri *et al.*, 2008). Asthma shares pathologies with other chronic inflammatory lung diseases such as chronic obstructive pulmonary disease, and fibrosis which include hyperreponsiveness of the airway, increased bronchial constriction (which is partly or fully reversible in asthma) and an increased airway wall thickness (Fabbri *et al.*, 2005). It has often been claimed that asthma is linked to atopy, but up to 50% of asthma patients have no proven allergies, and the percentage varies with age, gender and lifestyle (Faniran *et al.*, 1999; London, 2007; Oryszczyn *et al.*, 2007).

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One of the most striking aspects of the pathology of the modified airway wall structure in asthma is the increased number and size of airway smooth muscle cells, which had already been reported by Huber and Koesser in 1922 (Huber and Koesser, 1922). Then, this smooth muscle bundle abnormality was considered to be the main cause of the airway hyperresponsiveness and the exaggerated constriction in asthma (Huber and Koesser, 1922). This idea was replaced by results of immunological studies which showed that the ratio of specific activated immune reactive cells infiltrating the lung changed from Th1 to Th2 phenotypes in patients with asthma (Kus *et al.*, 1985; Cohn *et al.*, 1999; Martinez and Holt, 1999; Kay, 2006). These findings were supported by animal models of asthma and the hypothesis that the cause of asthma is a deregulated response of the immune system initiated by environmental factors became accepted (Hofstra *et al.*, 1998; Randolph *et al.*, 1999; Schramm *et al.*, 2003; Vermaelen and Pauwels, 2003). Except for a few studies (Kumar *et al.*, 2002; 2004), most mouse models of asthma reproduced the inflammatory aspect of the disease and ignored the increased airway wall remodelling so typical of asthma. An update of the Global Strategy for Asthma Management and Prevention Report from 1995 (GINA) confirmed the definition of asthma as a disease of activated mast cells, eosinophils and T-lymphocytes in the lung which lead to recurrent episodes of wheezing, breathlessness, chest tightness, cough and partly reversible airflow obstruction (Bateman *et al.*, 2008). In summary, it is widely accepted that chronic inflammation of the lung causes an increase in airway responsiveness and remodelling.

In line with the concept of inflammation as the cause of asthma is one of the earliest unquestioned findings in asthma research: a genetic predisposition (Le Souëf *et al.*, 2006; London, 2007; Oryszczyn *et al.*, 2007; Scirica and Celedón, 2007), which is often associated with elevated immunoglobulin E (IgE) and interleukin (IL)-4 levels (Battle *et al.*, 2007; Mak *et al.*, 2007; Chatterjee *et al.*, 2008; Inoue *et al.*, 2008). However, the link between IgE and IL-4 polymorphisms and asthma is uncertain as there is wide variability among ethnic groups (Le Souëf *et al.*, 2006; Scirica and Celedón, 2007). Then, there are re-occurring reports linking the asthma predisposition to a maternal inheritance, which was often further associated with the effect of hormones (Kuiper *et al.*, 2006; Bjerg *et al.*, 2007; Raby *et al.*, 2007; Barrett, 2008). Interestingly, one study provided a correlation of the inheritance of asthma susceptibility to mitochondrial driven genes, including IgE (Raby *et al.*, 2007). This possibility will be discussed in more detail later. Other studies suggested asthma to be due to mutations of the β_2-adrenoceptor (Broadley, 2006; Yang *et al.*, 2007), the glucocorticoid receptor (Hawkins *et al.*, 2004; Stevens *et al.*, 2004) and of proteases such as the metalloproteinase/chitinase ADAM33 (Kedda *et al.*, 2006; Foley *et al.*, 2007). The latter has recently been linked to other chronic inflammatory diseases (Wjst, 2007). Thus, there are many candidate genes which correlate with asthma susceptibility, but none can fully explain the various pathologies of asthma.

Interestingly, an increasing number of studies points back to the pathologic airway smooth muscle cell as a major cause of asthma. What properties of the airway smooth muscle cell would support this idea? Studies of childhood asthma showed that the increased mass of airway smooth muscle exists already in very young children and does not necessarily correlate with the severity and duration of the disease as it was assumed earlier (Cutz *et al.*, 1978; Cokugras *et al.*, 2001; Jeffery, 2001; McKay and Hogg, 2002; Jenkins *et al.*, 2003; Payne *et al.*, 2003). Furthermore, airway inflammation is not present in all patients with childhood asthma, while remodelling is (Jeffery, 2001; Lex *et al.*, 2006). Human and animal studies in childhood asthma suggest that the capability of the airway to relax correlates with the fast growth of the lung, and therefore with less differentiated cells which proliferate and produce proinflammatory mediators (Chitano and Murphy, 2003; Chitano *et al.*, 2005; Plopper *et al.*, 2007). This is an interesting observation as it fits with the lower expression of the transcription and differentiation factor CCAAT/enhancer binding protein (C/EBP)-α that we described in asthmatic airway smooth muscle cells *in vitro* and in *ex vivo* experiments (Borger *et al.*, 2002; 2007; Roth *et al.*, 2004). However, this finding has not yet been validated in childhood asthma, only in cells of adult asthma patients.

The most terrifying experience of asthma patients is the sustained constriction of the airway smooth muscle which narrows the lumen of the airways and makes breathing difficult. The mechanism by which the constriction of the airway smooth muscle bundles is triggered not only by so many different factors including inhaled plant pollen, animal hair, dust, food, but also by cold, or humid air, or other natural occurring compounds such as salicylic acid is not understood and cannot be explained by an overreactive immune system (Kariyawasam *et al.*, 2007; Holgate, 2008).

It has been shown by us and others that isolated airway smooth muscle cells of asthmatic patients contract more forcefully and their relaxation is slower compared with non-diseased cells (Ma *et al.*, 2002; Stephens *et al.*, 2003; Matsumoto *et al.*, 2007). The slow relaxation of airway smooth muscle bundles in asthma was correlated to the increased thickness of the airway wall, which in addition to more muscle results from: an increase in the thickness of the basement membrane, more myo-fibroblasts and the increased extracellular matrix deposition (Johnson *et al.*, 2006; Bossé *et al.*, 2008; Moir *et al.*, 2008). The stiffness of the airway walls in asthma may result solely from the modified composition of the extracellular matrix (Dekkers *et al.*, 2007; Slats *et al.*, 2008; Zhang and Gunst, 2008), which in addition may further stimulate the infiltration of circulating eosinophils into the lung via integrins and collagen receptors (Bazan-Socha *et al.*, 2006; Moir *et al.*, 2008) or eotaxin (Chan *et al.*, 2006). These changes in the composition of the airway wall extracellular matrix may furthermore explain why the muscle relaxation after an asthma attack is incomplete, as it would not be due to muscle constriction alone. The loss of tissue water through enhanced pressure on the airway wall as it occurs during an asthma attack would increase the stiffness of the airway wall and would interfere with the recovery of the muscle constriction.

The best way to relieve the airway constriction is the inhalation of β_2-agonists (Barnes, 2002). The function of β_2-agonists seems to be restricted to the fast relaxation of the constricted airway muscle, while glucocorticoids inhibit the inflammation.

Glucocorticoids act mainly at the level of gene activity: (i) as negative transcription factors suppressing gene promoter activity; (ii) reducing the unwinding of the deoxyribonucleic acid (DNA)/histone complexes thereby hindering the binding of transcription factors especially of nuclear factor kappa B; and (iii) by binding other activated transcription factors in the cytosol and modifying their DNA binding specificity (Barnes, 2006).

Similarly, other classes of anti-asthmatic drugs such as anti-IgE antibodies, phosphodiesterase inhibitors or leukotriene receptor antagonists dampen the inflammatory response of the immune system in asthma, thereby relieving the symptoms (Giembycz, 2008; Hanania, 2008; Montuschi, 2008; Prenner, 2008). However, none of the available drugs today significantly affects or reverses the airway wall remodelling. On the contrary, there is evidence that glucocorticoids may worsen airway remodelling under certain conditions in humans at least *in vitro* (Chakir *et al.*, 2003; de Kluijver *et al.*, 2005; Goulet *et al.*, 2007), but not in animal models (McMillan *et al.*, 2005). Such species-specific differences may be explained by a feedback mechanism between collagens and glucocorticoid signalling (Bonacci *et al.*, 2003; Goulet *et al.*, 2007).

The question as to whether the airway smooth muscle cell contributes to inflammation can be clearly answered with 'Yes'. In animal models and in humans, it has been demonstrated that isolated airway smooth muscle cells can release a wide range of pro-inflammatory cytokines tumour necrosis factor-α, IL-1β or IL-6, as well as various cytokines and/or chemokines IL-4, IL-8, IL-12, stem cell factor, tumour growth factor (TGF)-β_1, inhibitory protein-10 and fractalkine which are well-known to activate and attract immune cells such as T-lymphocytes, mast cells or neutrophils into the lung (Berger *et al.*, 2003; Brightling *et al.*, 2005, El-Shazly *et al.*, 2006; Doherty and Broide, 2007). Human isolated airway smooth muscle cells of asthma patients also release and respond to growth factors that stimulate the synthesis and deposition of extracellular matrix (connective tissue growth factor, TGF-β) which would further contribute to the increased thickness of the airway wall (Burgess *et al.*, 2006). It was also shown that mechanical stress of airway smooth muscle cells, as would occur during an asthma attack, triggers *de novo* microvascularization which is well documented in the lamina propria in asthma patients. This effect depends on the release of vascular endothelial growth factor (Hasaneen *et al.*, 2007; Simcock *et al.*, 2008). Furthermore, T-lymphocytes and macrophages, under certain conditions, adhere to airway smooth muscle cells and stimulate the inflammatory response (Lazaar *et al.*, 1994; Ramos-Barbón *et al.*, 2005). In summary, these data suggest that the airway smooth muscle cell significantly contributes or regulates local airway inflammation. An overview of the possibilities as to how airway smooth muscle cells can contribute to the characteristic pathology of asthma is provided in Figure 1.

The increased number of airway smooth muscle cells in asthma was the first reported histological airway pathology (Huber and Koesser, 1922). We were the first to show that isolated human airway smooth muscle cells proliferate faster compared with cells of non-asthmatic airways (Johnson *et al.*, 2001; Roth *et al.*, 2004) and this finding was recently confirmed by an independent study (Trian *et al.*, 2007). However, there is a recent study that could not confirm this pathology of airway smooth muscle cells neither *in vivo* nor *in vitro* (Ward *et al.*, 2008). The disagreement between these studies can easily be explained. First, it cannot be assumed that the cellular turnover of airway smooth muscle cells is significantly higher in established asthma as Trian *et al.* and we provided evidence that the cells only proliferate faster under certain conditions. Second, the fact that Ward *et al.* did not observe increased proliferation *in vitro* may be due to the much shorter time window they used to examine proliferation. The faster proliferation of asthmatic airway smooth muscle cells only became significant after at least 3 days of culture (Johnson *et al.*, 2001; Borger *et al.*, 2007; Trian *et al.*, 2007), while the maximal observation period in the study by Ward *et al.* (2008) was 2 days.

There is a much more impressive argument for the initiating role of airway smooth muscle cells in asthma which comes from a novel type of therapy, the removal of the airway smooth muscle cells by bronchoscopic hyperthermia (Brown *et al.*, 2005; Cox *et al.*, 2006). Not only in animals, but recently in humans, this new therapy resulted in a significant and lasting improvement (3 years) of all clinical asthma symptoms, including significantly lower airway hyperresponsiveness to experimental challenges, fewer hospital admissions, decreased requirement for inhaled anti-asthma drugs and an overall improved quality of life (Cox *et al.*, 2007). However, hyperthermia as a therapy seems to be a very radical method and therefore other possibilities to re-adjust the behaviour of airway smooth muscle cells should be found.

The basis for such a modification of airway smooth muscle biology may come from a combination of the inadequate expression of C/EBP-α (Roth *et al.*, 2004; Borger *et al.*, 2007) and overactive mitochondria (Trian *et al.*, 2007). First, the lowered expression of C/EBP-α seems to be due to a lack of proper translation, as we also observed reduced levels of the translation controlling factor eukaryotic initiation factor-4E (eIF-4E) in airway smooth muscle cells of asthma patients (Borger *et al.*, 2009). As eIF-4E is the end-point of the mammalian target of rapamycin (mTOR) signalling cascade, a deregulation of this factor, or of any upstream factor, may down-regulate C/EBP-α expression. Furthermore, this may link the C/EBP-α deficiency to nutrition, lipid, vitamin and calcium metabolism, all of which have been linked to asthma (Raught *et al.*, 2004; Lian *et al.*, 2008). Modulation of the translation of C/EBP-α in asthma patients may provide a novel therapeutic strategy which may cure the disease; however, none of the existing asthma drugs exhibit this property (Roth *et al.*, 2004; Borger *et al.*, 2007).

Second, the diminished expression of C/EBP-α may also modify the activity and function of mitochondria which was reported by Trian *et al.* (2007). At this stage, it cannot be concluded that the increased number of mitochondria and the increase in mitochondria-specific transcription factors in asthmatic airway smooth muscle cells is due to the reduced expression of C/EBP-α, but the increased activity of mitochondria (Trian *et al.*, 2007) may be linked to this. Mitochondria are the cell's major regulators of respiration and control the cell's energy use. They contain their own maternally inherited genes (Hood *et al.*, 2006; Scarpulla, 2008) and thus may present the link to the maternal inheritance of asthma

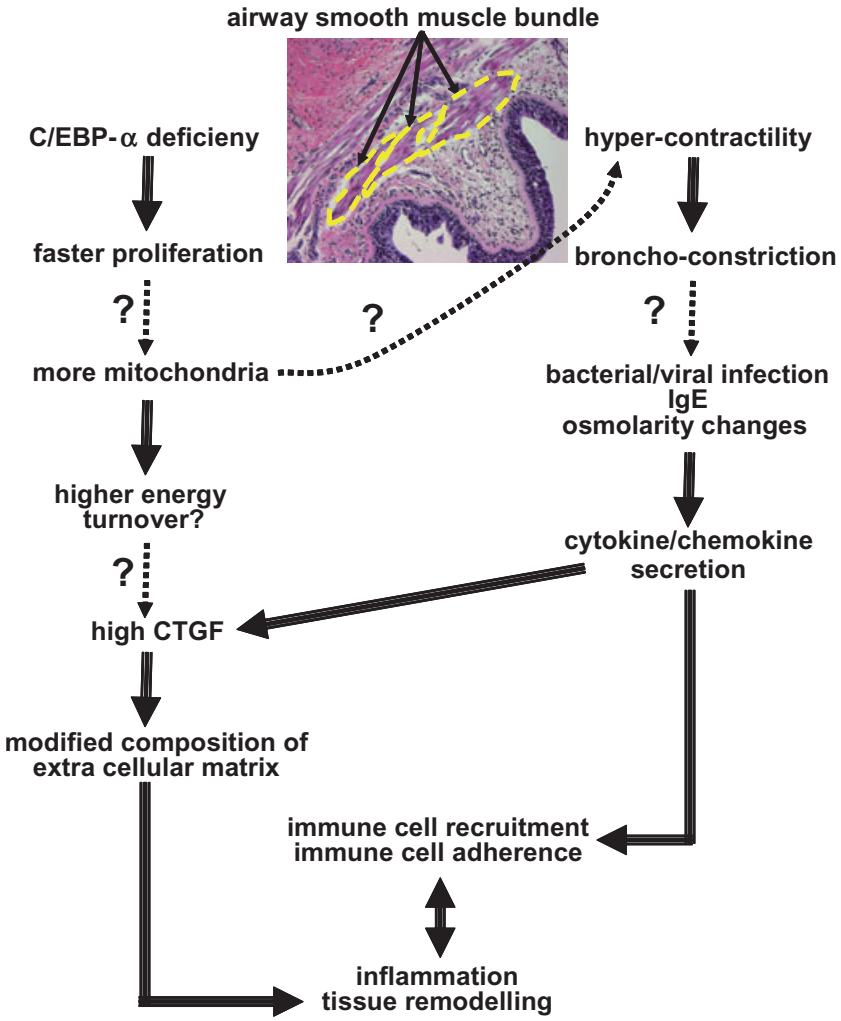

Figure 1 The contribution of airway smooth muscle cells to the pathology of asthma. Dotted arrows with a question mark indicate that this possible link is hypothetical at this stage. C/EBP, CCAAT/enhancer binding protein; CTGF, connective tissue growth factor; IgE, immunoglobulin E.

susceptibility (Kuiper *et al.*, 2006; Bjerg *et al.*, 2007; Raby *et al.*, 2007; Barrett, 2008). Moreover, in an increasing number of diseases, mutations of mitochondrial genes that can affect nutrient metabolism, oxygen sensor systems, carcinogenesis and regulate longevity (Crimi and Rigolio, 2008; Michelakis, 2008).

How does this link to the C/EBP-α deficiency in asthmatic airway smooth muscle cells? A significant number of the approximately 76 known human mitochondrial genes (Wallace *et al.*, 1995) contain a potential C/EBP binding site. However, the promoter structure of most mitochondrial genes is not well studied. Interestingly, some of these genes are regulated by a unique, bidirectional functioning promoter (Zhao *et al.*, 2002; Zanotto *et al.*, 2007). Unfortunately, the mechanism which directs the site-specific action of the transcription complex is not understood. Genes with a known C/EBP controlled bidirectional promoter are mitoribosomal protein S12 and mitochondrial seryl-tRNA ligase, which both control mRNA translation (Lopez *et al.*, 2001) Chaperonin 60 (mtDnaJ, Hsp60) and chaperonin 10 (ClpP, Hsp10) which are essential regulators of protein folding including the folding of the glucocorticoid receptor (Silverman *et al.*, 2006). Other mitochondrial genes which are regulated by C/EBP binding sites are: mitochondrial 3-hydroxy-3-methylgluatryl CoA reductase (Sugiyama *et al.*, 2001), steroidogeneic acute regulatory protein D (Ericsson *et al.*, 1997), serine : pyruvate/alanie: glycoxylate aminotransferase (Yubero *et al.*, 1994), glycerol-3-phosphate acyltransferase (Hattori *et al.*, 2007), mitochondrial brown fat uncoupling protein (Helander *et al.*, 1997), phospholipid hydroperoxide glutathione peroxidase (Stankov *et al.*, 2007) and 2,4-dienoyl-CoA reductase (Saks *et al.*, 2007). Whether these genes are under the control of bi- or unidirectional promoters is unknown. In other cell types, it was indicated that the number of mitochondria directly correlates to the differentiation stage (Stankov *et al.*, 2007). In regard to asthma and muscle cell function, it might be important to note that, in skeletal muscle cells, the mitochondria are the main supplier and controller of exercise induced cell-type-specific nutrient metabolism and of cell response to exercise and stress (Stankov *et al.*, 2007; Kukat and Trifunovic, 2008; Yi *et al.*, 2008). An overview of the possible contribution of mitochondria to asthma pathology is provided in Figure 2.

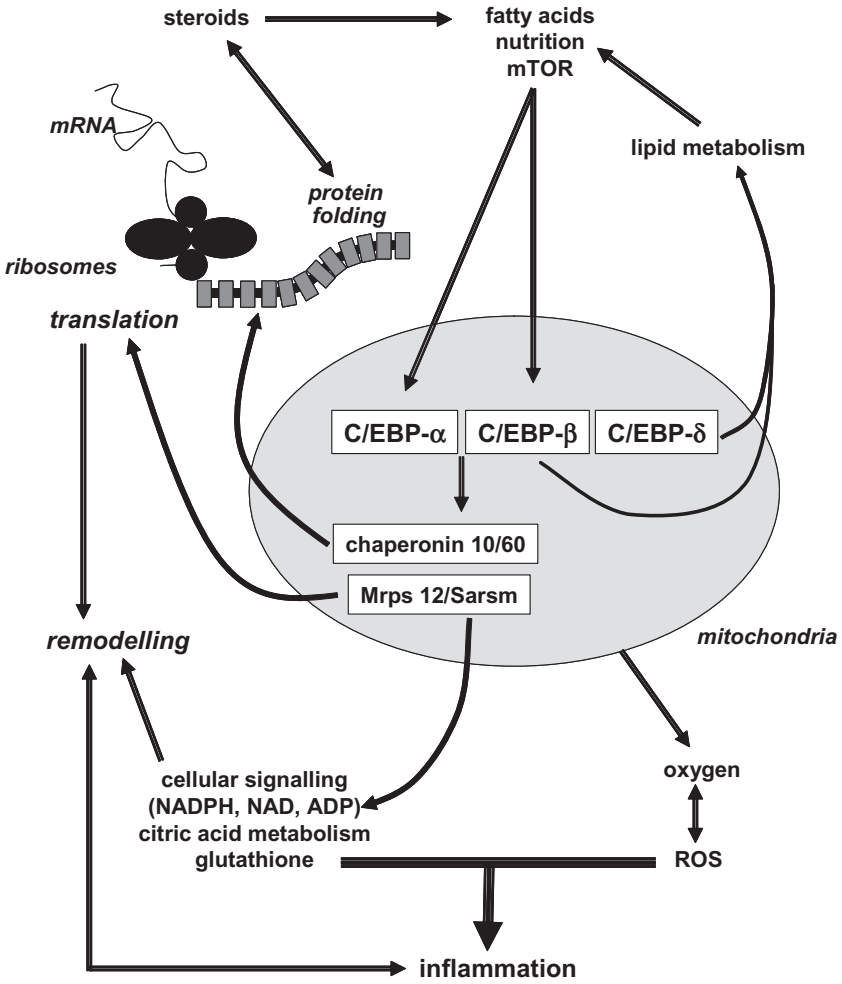

Figure 2 The possible impact of dysregulated mitochondria on asthma and their regulation by C/EBP-isoforms. ADP, adenosinediphosphate; C/EBP, CCAAT/enhancer binding protein; mTOR, mammalian target of rapamycin; NAD, nicotinamidadenindinucleotid; NADPH, nicotinamidadenindinucleotidphosphate; ROS, reactive oxygen species.

Therefore, we hypothesize that a deregulated translation of C/EBP-α mRNA and the overactivity of mitochondria in airway smooth muscle cells in asthma patients are causatively linked. If this theory can be substantiated, it will open new therapeutic approaches for asthma and may even raise the possibility of a cure for the disease. Potential new asthma drugs may be found in the signalling pathway controlling the mTOR-related translation mechanism; however, existing drugs inhibit mTOR instead of inducing it. The second option would be to find new mitochondria-regulating substances which reduce their activity overall or that of only specific proteins.

Conclusion

Increasing clinical and experimental evidence suggest a significant contribution or even a causative role of the airway smooth muscle cell in the pathology of asthma. In this review we made the attempt to link the most recent findings of molecular pathologies in airway smooth muscle cells of asthma patients. The data indicate that in asthma there exists a cell-type-specific deregulation of cell differentiation factors, especially the transcription factor family of C/EBPs, peroxisome proliferator-activated receptors (PPARs) and an up-regulation of mitochondria. Each factor alone is able to enhance the predisposition of muscle cells to proliferate, to produce more matrix and to release more chemoattractive cytokines, which inturn activates the immune system. Unfortunately, these factors closely control each others function and at this stage it is impossible to conclude which of them is causative to asthma. Moreover, the pathologic expression of all these factors can be regulated by inhaled allergens and other asthma provoking agents or conditions. Nevertheless, the available data open up novel approaches to develop new therapeutic strategies which will do more than just control the symptoms. New targets may be the re-adjustment of C/EBP-isoform expression and their interaction with the glucocorticid receptor, or with the PPARs, or the adjustment of mitochondria multiplication, or the better control of their activity.

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Conflicts of interest

None.

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