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Impact of Physical Activity and Doping on Epigenetic Gene Regulation

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To achieve success in sports, many athletes consume doping substances, such as anabolic androgenic steroids and growth hormones, and ignore the negative influence of these drugs on their health. Apart from the unethical aspect of doping in sports, it is essential to consider the tremendous risk it represents to their physical condition. The abuse of pharmaceuticals which improve athletic performance may alter the expression of specific genes involved in muscle and bone metabolism by epigenetic mechanisms, such as DNA methylation and histone modifications. Moreover, excessive and relentless training to increase the muscle mass, may also have an influence on the health of the athletes. This stress releases neurotransmitters and growth factors, and may affect the expression of endogenous genes by DNA methylation, too. This paper focuses on the relationship between epigenetic mechanisms and sports, highlights the potential consequences of abuse of doping drugs on gene expression, and describes methods to molecularly detect epigenetic changes of gene markers reflecting the physiological or metabolic effects of doping agents.

Keywords: DNA methylation; histone modifications; growth hormones; IGF-1

Introduction

Rapid growth and development of muscles by high physical activity is based on an integrated mechanism linking exercise with a variety of anabolic responses. The physical adaptation to environmental conditions, such as strongly developed muscles and tendons and a more resilient blood circulation, might influence epigenetic mechanisms. The so-called eustress in sports releases neurotransmitter and growth factors which may cause a change in gene expression by epigenetic mechanisms. The effects of environmental exposures can even be transmitted for several generations, suggesting transgenerational inheritance of induced epigenetic variation.[1] The illegal intake of anabolic substances can affect the body-own gene activity in the form of an activation or repression of gene expression. The most frequently used doping agents are growth hormones and anabolic steroids that are readily detectable in urine using gas chromatography and mass spectrometry.[2–4] It is estimated that every fourth sportsman takes combinations of anabolic steroids and IGF-1 (insulin-like growth factor 1). Although it is little known about the long-term effects on gene expression, these substances could influence the epigenetic pattern of the genome.[5]

Epigenetics covers three molecular mechanisms: DNA methylation, histone modifications, and remodulation of the chromatin.[6] These processes control the transcription of genes responsible for the variability of the phenotypes. DNA methylation affects directly or indirectly the gene expression. In general, DNA methylation inhibits the access of factors to their target sequences on promoters and introns (e.g. enhances, silencer), and thus, leading to the inactivation of transcriptional expression. Moreover, the degree of chromatin condensation has a regulatory role on gene expression. In this regard, switching on and off distinct sets of genes to achieve lineage-specific activation or repression could, hence, improve the physical activity.[6] It may be speculated, that drug-induced long-lasting changes of gene expression can increase the risk of cancer.[7] Therefore, increasing attention should be paid to the role of DNA methylation and histone modifications in doping. To date, a main challenge of World Anti-Doping Agency (WADA) and International Olympic Committee (IOC) is to prevent serious health risks and reduce unfair competitive advantage among athletes. Hence, specific, sensitive, and validated methods are needed or should be adapted to the sport setting, in order to document the modulated gene expression profile underlying the epigenetic influences of doping.

DNA methylation

DNA methylation is a natural form of DNA modification. This epigenetic process occurs by the addition of methyl groups (CH_3) to DNA molecules and is mediated by DNA methyltransferases. With exception of Saccharomyces cerevisiae und Caenorhabditis elegans, DNA methylation seems to be present in nearly all animals.[8] In mammals, the only known modification of DNA is the methylation of the C5 position of nucleotide cytosine within the dinucleotide CpG. Approximately 1% of the human genome contains CpG-rich sequences (CpG islands) with a size of 0.5–4 kb. Half of these CpG islands are located in the proximal promoter region of genes controlling the transcriptional expression. To a large extent, these regulatory sequences are unmethylated.[9] The tissue-specific methylation pattern is defined during the cell development and differentiation, and is passed to the somatic cells.[10] It is commonly accepted that an equilibrium exists between the reversible processes of DNA methylation and demethylation.[11] In general, DNA methylation contributes

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to the inactivation of gene expression and thus, to the gene regulation. This process affects, for example, development, genomic imprinting, and inactivation of the female X chromosome, transposable elements and cancer testis genes,[8] and possibly affects the physical activity. Conversely, DNA demethylation leads to the activation of gene expression.

To date, four biologically active DNA methyltransferases, which add methyl groups to the cytosines, are known. The DNA methyltransferases 3A and 3B are de novo methylases, while DNA methyltransferase 1 ensures preservation of the cellular methylation pattern. During the cell division DNA methyltransferase 1 uses the methylated, parental DNA strand as matrix established by DNA methyltransferases 3A and 3B, and passes the existing methylation pattern to the following cell generations.[12,13]

Histone modifications

Not only DNA methylation is involved in regulation of gene transcription, but also configuration changes in the nuclear chromatin structure. A chromatin thread is a flexibly connected chain of nucleosomes. The individual nucleosomes consist of approximately 200 bp-long DNA and two histone molecules of each H2A, H2B, H3 and H4. The histones form a central octamer core, around which DNA is wrapped approximately twice. The packing of DNA is modulated by post-transcriptional modifications at the amino-terminal tail of the histones, such as histone acetylation, methylation, phosporylation, ribosylation, and ubiquitination. These histone modifications are mediated by specific chromatin-modifying enzymes.[14,15] Acetylation of lysine and phosphorylation of serine may be predominant for active chromatin through neutralizing the negative charges of DNA and unfolding the chromatin structure, whereas methylation of lysine condenses the structure.

Repressor complexes which possess histone deacetylase, histone methyltransferase or chromatin modulation activity are involved in the molecular mechanism underlying the association between chromatin architecture and DNA methylation. CpG binding domain proteins (MBDs), which interpret the methylation pattern, recruit repressor complexes to methylated DNA and contribute to the formation of a compact and transcriptional inactive chromatin structure.[7] Thus, DNA methylation together with histone deacetylation and methylation may inactivate promoters, and both mechanisms play a crucial role in the repression of the gene transcription.

Growing body of evidence suggests that epigenetic mechanisms regulating gene repression, such as DNA methylation and chromatin modifications, are influenced by the environment. It was reported that changes in the environmental conditions can modify histone signature in the nucleus.[16] After exposure to a forced swim test, exercised rats presented improved coping responses and memory performance to their novel environment. It seemed that the exercised animals were much quicker than their sedentary counterparts in assessing the novel situation. This better adaptive capacity has been attributed to the complex of elevated cognitive abilities, lowered anxiety levels, and decreased impulsiveness known of exercised subjects. The improved trainability of the rats was associated with enhanced responses in phosphoacetylation of histone H3 and the induction of the transcription factor c-Fos in dentate granule neurons.[16]

Influence of nutrition on DNA methylation

In cells the main donor of DNA methylation process is S-adenosyl methionine (SAM), which is synthesized directly from the amino acid methionine. Two cofactors, vitamin B12 and folic acid, play an important role in the synthesis of methionine. Varying quantities of these cofactors may lead to an increase or reduction of cellular DNA methylation. Subtherapeutic administration of vitamin B12 strongly limits the formation of SAM. Likewise, mutations in the methionine synthase lead to a substantial change in the methylation pattern concerning all processes of methylation (protein, RNA, and DNA) which use SAM as a methyl group donor.[17–19] These observations indicate that not only defects on enzyme level but also the direct influence of nutrition correlate with a change in the DNA methylation pattern. Particularly, due to their function in the erythrocyte formation, folic acids and vitamin B12 are of special interest in sport-medical view, since high endeavours in training may attribute to a lack of these substances.[20]

Bioactive dietary components, such as genistein (soybean), tea polyphenols (green tea), resveratrol (grapes), and sulforaphane (cruciferous vegetables) may have an impact on DNA methylation. Previously, it was reported[21] that maternal dietary genistein supplementation for mice during gestation, at levels comparable with humans consuming high-soy diets, shifted the coat colour of heterozygous viable yellow agouti offspring towards pseudoagouti. The marked phenotypic change was significantly associated with increased DNA methylation of a retrotransposon upstream of the transcription start site of the Agouti gene. This genistein-induced hypermethylation acts during early embryonic development and persisted into adulthood, decreasing ectopic Agouti expression and protecting offspring from obesity. Thus, these investigations revealed that in utero of mice dietary genistein affects gene expression and alters susceptibility to obesity in adulthood by permanently altering the epigenome.[21]

Alteration of the global DNA methylation pattern in tumorigenesis

Global chromatin changes have shown to occur in tumours. In regions of tumour-associated genes, epigenetic modifications may constitute important regulatory mechanisms for the pathogenesis of malignant transformation.[22] In tumour development and progression, promoter hypermethylation of tumour suppressor genes is frequently detected, leading to their transcriptional inactivation.[23] Gene products of tumour suppressor genes have important functions in the processes of DNA repair, cell cycle control or apoptosis.[9,24] Not only the gene silencing determined by DNA methylation may contribute to tumorigenesis, but also methylated cytosines have an oncogenetic character. In somatic cells, approximately 50% of the inactivating point mutations of tumour suppressor gene p53 are generated by methylated cytosines. For example, the spontaneous desamination of methylated cytosine results in transition of thymidin.[25] However, not only promoter hypermethylation is associated with the tumorigenity of cells, but also the loss of a cell-specific DNA methylation pattern. In the year 1983, it was shown that the genome in cancer cells is less strongly methylated than in comparable normal, *wild type* cells.[26] DNA hypomethylation is a very frequent and early event during cancer development, and is to be found primarily in repetitive regions of the genome. In larger DNA sections it

may cause chromosomal instability, and promote DNA translocation, deletion, inversion, and amplification. Furthermore, DNA demethylation leads to an increased expression of oncogenes.[27] The demethylation of promoters of transposable elements leads to a reactivation of transposomes and results into an aberrant gene regulation which is mediated by the formation of antisense transcripts.[27]

Techniques for detection of DNA methylation and strategies for induction of silenced genes

The DNA methylation status of genes can be analyzed by various techniques, for example, sodium bisulfite sequencing, methyl-sensitive PCR, chromatin immunoprecipitation or the use of DNA methylation array microchips.

For the exact determination of DNA methylation patterns, sodium bisulfite sequencing is usually carried out. Extracted genomic DNA is denatured and treated by sodium bisulfite. Following precipitation and resuspension, the modified DNA is amplified with gene-specific primers. Since sodium bisulfite converts unmethylated cytosine residues into uracil, in contrast to methylated cytosine, the methylation pattern can be determined by DNA sequencing.[28]

For the methyl-sensitive PCR, isolated genomic DNA is digested by the methylation-sensitive and methylation-insensitive restriction enzymes HpaII and MspI, respectively. The digested DNA is amplified with gene-specific primers. A PCR product is only available if the promoter of the gene is methylated and consequently not cleaved.[28]

The chromatin immunoprecipitation is performed to detect whether proteins (e.g. MBDs) bind to methylated DNA. In this method, sonicated chromatin fragments cross-linked to DNA are immunoprecipitated using protein-specific antibodies and protein G beads. After reversion of the protein-DNA crosslinks, purified DNA is amplified by PCR with gene-specific primers.[29]

DNA methylation microchips contain numerous DNA probes. The hybridization of extracted DNA and competitors to DNA probes and the subsequent data evaluation of the signals deliver information on the methylation profile of a large number of selected genes.

For the induction of silenced genes, cells are incubated by cytidin analogues and histone deacetylase inhibitors. The cytidin analogues are built into DNA daughter strands during the cell division and the associated DNA replication. The DNA integration of the cytidin analogue leads to an irreversible inhibition of the DNA methyltransferase 1 and in the next cell generations to DNA demethylation. This technology uses the proliferating characteristics of cells. The histone deacetylase inhibitors induce the hyperacetylation of nucleosomal histones. The strong reciprocal effect between negatively charged DNA and positively charged histones is abrogated by the administration of these inhibitors leading to decondensation of the chromatin structure. Thus, DNA becomes more accessible for transcription factors, which are necessary for the gene expression.[28]

Epigenetic impact on the regulation of myogenesis

Skeletal muscles are derived from the mesoderm and consist of multinucleated myofibres. Since the nuclei of myofibres are post-mitotic, growth and regeneration of skeletal muscles depend on a pool of satellite cells, the stem cells of adult skeletal muscles. Satellite cells reside under the basal lamina of myofibres and are usually quiescent. In response to stimuli, such as injury or physical exercise, they are activated to proliferate and differentiate.[30] A series of transcriptional controls governed by skeletal muscle-specific regulatory factors including Myf5, MyoD, myogenin and MRF4 are involved in this myogenic differentiation pathway (myogenesis). MyoD and MEF2 recruit enzymes that introduce post-translational histone modifications, which allow induction or repression of further muscle-specific genes.[31] The treatment of embryonic fibroblasts with a DNA methyltransferase inhibitor, 5-azacytidine, converts these cells to muscles leading to demethylation of the CpG island surrounding the MyoD promoter. This relationship between DNA demethylation and the activation of the myogenic program shows that MyoD in satellite cells is methylated.[32] Moreover, this finding reveals the epigenetic impact on the regulation of myogenesis.

The muscular adaptation process to a prolonged training causes the gene expression of myogenic molecules and hence, the activation, and proliferation of the satellite cells. After a single session of training myogenin, MyoD, and MRF4 mRNA levels are transiently elevated in skeletal muscle.[33] Thus, physical exercise can alter skeletal muscle mRNA expression of several genes associated with muscle growth and remodelling. After physical loading, unmethylated MyoD may be detected in the blood circulation of sportspersons and allows speculations on the cellular origin of the released DNA. However, this hypothesis has to be tested.

Abuse and effect of anabolic substances

Growth hormone (GH) regulates important physiological processes, including somatic growth and development as well as muscle protein synthesis and carbohydrate and lipid metabolism, directly by binding to and activation of its receptors or indirectly by stimulation of IGF1. Several pathways contribute to initiate myogenesis in response to different extracellular signals. The PI3K/Akt pathway, activated by IGF-1, the stress activated MKK6/p38 MAPK pathway and the calcium/calmodulin-dependent kinase (CaMK) pathway promote muscle-specific gene expression. In myogenesis, IGF-1 and myostatin are key signalling molecules and control skeletal muscle mass. IGF-1 is a positive and myostatin is a negative regulator.[34] IGF-1 induces satellite cell proliferation, myoblast differentiation and subsequent myoblast fusion into myotubes. Knock-out mice of IGF-1 or its GH receptor show increased growth retardation and reduced organ and skeletal muscle mass,[35] while overexpression of IGF-1 within skeletal muscle causes significant myofibre hypertrophy and myoblast proliferation.[36] Investigational studies have demonstrated that acute exercise increases circulating IGF-1 levels in blood.[37] On the other hand, mice that lacked myostatin, a member of the transforming growth factor β superfamily of secreted growth and differentiation factors, were found not only to be stronger and more muscular than their counterparts with normal myostatin levels, but also to have reduced fibrosis and fatty remodelling, suggesting improved regeneration of muscle.[38]

Abuse of GH is widespread; overdose of GH leads to increased muscle protein synthesis along with increased fatty acid availability and sparing of glycogen stores. Most of these anabolic effects of GH are mediated by IGF-1.[39] Thus, based on their attributes

which make GH and IGF-1 attractive as potential performance-enhancing agents, they are often used as doping substances by athletes. Approximately every fourth sportsman who uses anabolic androgenic steroids also takes these both drugs.[40] As measured by maximal oxygen uptake, the combination of GH and testosterone improves the fitness, which is larger than with one compound alone.[41] Anabolic androgenic steroids stimulate hypertrophy and mass of muscle fibres by regulation of satellite cell proliferation, the number of myonuclei and the balance of muscle proteins.[42] The exact biochemical mechanisms responsible for increased skeletal muscle mass and strength by anabolic androgenic steroids are partly unknown. In myoblast culture systems, testosterone has been shown to be able to stimulate the mitotic activity of satellite cells, and IGF-1 signalling pathway plays an important role in mediating these effects of testosterone on skeletal muscle cell growth and differentiation.[43] Following anabolic steroid administration, increased expression of IGF-1 together with alterations in expression of several IGF-1 binding proteins has been observed in muscle.[42] Hence, at least in part the muscle IGF-1 seems to mediate the growth-promoting influences of anabolic steroids.

Epigenetic influences of GH and IGF-1

Up-regulation of GH in hepatic chromatin induced rapid and dramatic changes at the IGF-1 locus and activated IGF-1 gene transcription by distinct promoter-specific mechanisms.[44] Alterations in the IGF-1 axis, which predispose individuals to diseases, have been reported to be due to an altered epigenetic regulation that can modify IGF-1 transcript levels.[44] Systemic GH treatment caused an approximately robust increase in transcription from each IGF-1 promoter within an hour of hormone administration and led to a sustained accumulation of IGF-1 mRNA. The coordinated induction of both IGF-1 promoters by GH was accompanied by hyperacetylation of histones H3 and H4 in promoter-associated chromatin, a decline in monomethylated lysine 4 of histone H3 (H3K4), and recruitment of RNA polymerase II to IGF-1 promoter 2.[44] These findings show that GH may regulate the transcript levels of IGF-1 by modifying the chromatin architecture.

The molecular mechanism underlying the improved muscle performance by the action of IGF-1 is not well understood. It is speculated that IGF-1 may modulate genomic methylation to promote normal development of muscles. Investigations on cell cultures showed that after a 6-hour exposure of cells, IGF-1 increased the global DNA methylation. This change in the nuclear methylation pattern is based on the fact that IGF-1 is able to stimulate the activity of methionine synthase via a mechanism that requires the activity of both PI3 kinase and MAP kinase pathways.[45] Moreover, during early embryonic development the occurrence of IGF-1 may affect the DNA methylation pattern allowing the activation of expression of particular genes important for embryonic survival.[46] Consequently, the administration of IGF-1 as doping agent might also affect DNA methylation in athletes. However, the long-term intakes of GH or IGF-1, along with their effects on DNA methylation signal transductions or modulations of gene expression, are currently unknown.

Doping and cancer

Due to the consensus statement of the Growth Hormone Research Society there seems to be no increased risk of cancer when GH is given at physiological replacement doses, but there is mounting evidence that the intake of GH higher than physiological doses for many years may be associated with increased incidences of colorectal, thyroid, breast, and prostate cancers.[47] In these cancer patients, high levels of circulating IGF-1 were detected in serum and have been associated with cancer risk and cancer prognosis.[47] Drug candidates that target IGF1 signal transduction have revealed anti-neoplastic activity. Moreover, the signalling pathway of IGF-1 is involved in tumour development and progression. Experiments have shown that cancer cells have an increased expression of IGF-1 receptors. Activation of IGF-1 receptor, an inducer of Akt and MAPK signalling networks, by IGF-1 has mitogenic and anti-apoptotic effects in neoplastic tissue.[48] Thus, IGF-1 may provide an anti-apoptotic environment that may favour survival of cancer cells.

Regarding the global chromatin changes during the malignant transformation of cells and the involvement of IGF-1 in DNA methylation, it is equitable to speculate that the increased abuse of GH and IGF-1 may at least partly contribute to the tumour-associated epigenetic changes.[48] However, too little data are available to sustain this theory. Future investigations are warranted to clarify the implication of DNA methylation in doping increasing cancer risk. Recent findings have indicated that IGF-1 may play a role in neoplasia. Although the extent of DNA methylation of IGF-1 gene was highly variable in most tumours as its mRNA expression levels, a relationship could be detected between IGF-1 overexpression and gene demethylation in hepatocarcinomas and tumours associated with hypoglycaemia.[49] In multiple myeloma IGF-1 mediated down-regulation of the proapoptotic BH3-only protein Bim by the activation of Akt and MAPK pathway. It has been reported that IGF-1 is involved in epigenetic regulation of the Bim promoter. Chromatin immunoprecipitation of IGF-1 showed both a reduced acetylation of lysine 9 of histone H3 (H3K9) and an increased H3K9 dimethylation, which contributed actively to the silencing of BIM. These data identified a new mechanism in the IGF-1 dependent survival of multiple myeloma cells.[50] Using chromatin immunoprecipitation assays, histone methyltransferase RIZ1 was shown to bind to the IGF-1 promoter and to increase histone H3 lysine 9 methylation.[51] In chronic myelogenous leukemia (CML), blastic transformation is associated with decreased expression and activity of RIZ1. Overexpression of RIZ1 in model CML blast crisis cell lines decreased proliferation, increased apoptosis and supported differentiation which was accompanied with a reduced IGF-1 receptor activation and activation of downstream signalling components ERK1/2 and AKT.[51] These data highlight the therapeutic potential of inhibiting IGF-1 signalling pathway by modulating the histone pattern at IGF-1 promoters. Possible intervention strategies including improvement of insulin sensitivity and reducing concentrations of IGF-1 in blood and tissue have also been suggested for preventing breast cancer.[52]

Conclusion

Physical alterations caused by sports, for example, increased muscle fibres by training or drug abuse, may induce a changed epigenetic profile leading to a changed gene expression. Doping control laboratories have begun to develop methods for detection of peptide hormones using electrophoretic, chromatographic, and mass spectrometric approaches. However, sensitive and specific techniques which reveal the molecular and epigenetic outcome

of doping agents are lacking. Thus, the effect of these substances on the genomic methylation pattern remains to be addressed. Therefore, efforts to translate currently available information on the molecular mechanisms of doping agents should be made.

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