Carnitine Enigma: From Antioxidant Action to Vitagene Regulation. Part 2. Transcription Factors and Practical Applications

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Abstract

A growing interest has been shown in the potential uses of carnitine in medical practice and animal/poultry industry. The molecular mechanisms accounting for the positive effect of LC on livestock animals are not yet fully understood but many protective effects of LC in various stress conditions reported in literature, have been related to its antioxidant action. Based on the analysis of the recent publications presented in the review it could be concluded that antioxidant actions of carnitine are associated to much extent with redox signaling in the cell. Indeed, LC is shown to upregulate Nrf2 and PPARs and downregulates NF-κB leading to anti-apoptotic and anti-inflammation actions of carnitine. In fact, Nrf2-mediated synthesis of antioxidant enzymes, including SOD, GSH-Px, GR, GST and GSH, in response to carnitine supplementation could be a main driving force of antioxidant action of carnitine and its derivatives. Furthermore, LC and its derivatives are shown to affect vitagene networks resulting in increased adaptive ability to stresses via additional synthesis of protective molecules, including heat shock proteins (HO-1), upregulating sirtuins, thioredoxins and SOD. It seems likely that in biological system in vivo the interactions of the aforementioned mechanisms provide an important place for carnitine to be a crucial part of the integrated antioxidant systems of the animal and human body. Furthermore, direct scavenging ROS and chelating properties of carnitine would be very much relevant to the antioxidant system of the gut. Taking into account low carnitine content in grains and poultry and pig diet formulations with limited amounts of animal proteins, carnitine requirement and possible inadequacy in commercial poultry and pig nutrition should receive more attention. Furthermore, protective roles of carnitine in stress conditions of commercial poultry and pig production, including its immunomodulating properties, are of great importance. Therefore, a development of carnitine-containing antioxidant compositions supplying via drinking water seems to be an important way forward in decreasing the detrimental consequence of various stresses in poultry and pig production.

Abbreviations

ALC: Acetyl-L-Carnitine; AREs: Antioxidant Response Elements; BSA: Bovine Serum Albumin; b.w.: Body Weight; CoQ: Coenzyme Q; eNOS: Endothelial Nitric Oxide Synthase; FFA: Free Fatty Acids; γGCS: γ-Glutamate Cysteine Ligase; GRα: Glucocorticoid Receptor-α; GR: Glutathione Reductase; GSH: Glutathione; GSH-Px: Glutathione Peroxidase; GST: Glutathione Transferase; HO: Heme Oxygenase; HSP: Heat Shock Protein; IL: Interleukin, ICAM1: Intercellular Adhesion Molecule 1; IFN-γ: Interferon Gamma; i.p.: Intraperitoneal; LA: Lipoic Acid; LC: L-Carnitine; L-NAME: N-nitro-L-arginine Methyl Ester; LPS: Lipopolysaccharide; MCP-1: Monocyte Chemoattractant Protein-1; MDA: Malondialdehyde; NF-κB: Nuclear Factor-kappa B; Nrf2: Nuclear Factor-erythroid-2-related Factor 2; NQO1- NAD(P)H: Quinone-oxidoreductase-1; PHA: Phytohemagglutinin; PHS: Pulmonary Hypertension Syndrome,

PGI2: Prostaglandin I2 (prostacyclin); PPARα: Peroxisome Proliferator Activated Receptor Alpha; RGCs: Retinal Ganglion Cells, ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; SOD: Superoxide Dismutase; TNFα: Trx-thioredoxins.

Introduction

For the last 30 years carnitine has received considerable attention in medical sciences and animal production due to its diverse functions and beneficial effects in various stress conditions. Carnitine functions in the body are diverse and include: a) transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix for oxidation and energy production; b) transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO_2 and H_2O in the Krebs cycle; c) modulation of the acyl-CoA/CoA ratio; d) storage of energy as acetyl-carnitine; e) modulation of toxic effects of poorly metabolized acyl groups by excreting them as carnitine esters; f) preservation of membrane integrity and mitochondria functions as well as apoptosis inhibition; g) participation in redox-signaling and vitagene activation; h) maintenance of the antioxidant systems of the body [1,2]. In the first part of the review [2] it has been clearly shown that antioxidant action of carnitine were, firstly, related to free radical scavenging and metal chelating, which could be relevant to the maintenance of the antioxidant-prooxidant balance in the gut. Secondly, and more importantly, carnitine participates in mitochondria integrity maintenance and prevents free radical formation in the electron transfer chain in mitochondria and inhibits activities of some ROS-generating enzymes. However, it seems likely that main antioxidant effect of carnitine is mediated via its participation in redox signaling

and transcription factor regulation as well as affecting vitagene networks. Therefore, the main aim of the review is to analyse possible roles of LC and its derivatives in regulation of transcription factors and vitagene networks with a special attention to possible practical applications of the protective carnitine properties in poultry production.

Oxidative Stress and Transcription Factors

Oxidation-reduction (redox) based regulation of gene expression is a fundamental regulatory mechanism in cell biology acting at the cell-signaling level. Since ROS are damaging to many biological molecules, the antioxidant systems are responsible for prevention of the damages. However, a basal level of oxidative stress is essential for cell adaptation and survival. Therefore, a moderate level of oxidative stress can create adaptive responses and improve adaptive ability to stressful challenges/conditions [3]. Indeed, in animals, redox-signaling pathways use ROS as signaling molecules to activate genes responsible for regulation of various functions including growth, differentiation, proliferation and apoptosis. Furthermore, the antioxidant defence systems are also under regulation by various transcription factors [4-7]. These pathways operate in coordinated manner being critically important for animal adaptation to various stresses. In particular, they include Keap1/Nrf2, NF-κB, PPARs, MAPK, AP1, etc. [8]. In recent years great attention has been paid to a basic leucine zipper transcription factor, Nuclear factor-erythroid-2-(NF-E2-) related factor 2 (Nrf2) and NF-κB.

Transcription factor Nrf2

It is known that Nrf2 is the redox-sensitive master regulator of oxidative stress signaling and oxidative stress responses and is critical for cell survival in stressful conditions [9]. It has been shown that the Nrf2 antioxidant response pathway is an important player in the cellular antioxidant defense. Indeed, it is responsible for activation of a variety of genes involved in early defence reactions of higher organisms [10,11]. High expression of Nrf2 in organs that face environmental stresses including lungs and small intestine [12] is a confirmation of its importance in stress adaptation processes. Clearly, Nrf2 has a significant role in adaptive responses to oxidative stress being involved in the induction of the expression of various antioxidant molecules to combat oxidative and electrophilic stress [13-16].

It is suggestive that under normal physiological conditions, Nrf2 is kept in the cytoplasm by forming an inactive complex with the negative regulator, Kelch-like-ECH-associated protein 1 (Keap1), which is anchored to the actin cytoskeleton. In fact, Keap1 sequesters Nrf2 in the cytoplasm and forwards it to a Cul3-based E3 ligase with the following rapid ubiquitin-proteasome degradation leading to a short (about 20 min) half-life of Nrf-2 under physiological conditions (for review see [17]). It seems likely that, Keap-1 is an important cellular redox sensor and upon exposure to oxidative or electrophilic stress, critical cysteine thiols of Keap1 are modified/oxidised and Keap1 loses its ability to ubiquitinate Nrf2 resulting in preventing its degradation. There are also other ways of Nrf2 activation. For example, phosphorylation of Nrf2 at specific serine and/or tyrosine residues also causes an Nfr2-Keap1 dissociation resulting

in Nrf2 release and translocation to nucleus, where it combines with a small musculoaponeurotic fibrosarcoma protein called Maf to form a heterodimer [18]. Indeed, by binding to the ARE in the upstream promoter region of genes encoding various antioxidant molecules, Nrf2 regulates the expression of antioxidant proteins, thiol molecules and other protective molecules. This includes enzymes of the first line of the antioxidant defence, namely SOD, GSH-Px and Catalase, detoxification enzymes (HO-1, NQO1, and GST), GSH-related proteins (γ-GCS), NADPH-producing enzymes and others stress-response proteins contributing to preventing oxidative and inflammatory damages [19,20]. In fact, hundreds of cytoprotective genes are regulated by Nrf2 [12] and gene products (proteins) are involved in the maintenance and responsiveness of the cellular antioxidant systems. Indeed, an orchestrated change in gene expression via Nrf2 and the ARE is a key mechanism of a protective effect against oxidative stress [21]. It is suggestive that, Nrf2 is controlled through a complex transcriptional/epigenetic and post-translational network that provides regulatory mechanisms ensuring Nrf2 activity increases in response to redox disturbances, inflammation, growth factor stimulation and nutrient/energy fluxes orchestrating adaptive responses to diverse forms of stress [22].

As mentioned above, there is a range of Nrf2 activating mechanisms, including stabilization of Nrf2 via Keap1 cysteine thiol modification and phosphorylation of Nrf2 by upstream kinases [23,24]. It is proven that effects of Nrf2 on the adaptive ability of cells is quite broad and is beyond activation of synthesis of antioxidant molecules. Indeed, Nrf2 also contributes to homeostasis by up-regulating the repair and degradation of damaged macromolecules, and by modulating intermediary metabolism conducting directs metabolic reprogramming during stress [20].

Recently molecular mechanisms of regulating roles of transcription factors in cellular adaptation to stress have been extensively studied. In particular, it has been suggested that low intensity oxidative stress is predominantly sensed by Keap1/Nrf2 system [8] with the following downstream up-regulation of the protective AO genes. It is interesting to note that intermediate oxidative stress also increases the activity of antioxidant enzymes, but mainly via NF-κB and AP-1 pathways [8]. Furthermore, at both, low and intermediate intensity oxidative stresses, MAP-kinases and other kinases seem to be involved in signal sensing and cellular response, leading to enhanced antioxidant potential [20]. Emerging evidence clearly indicates that Nrf2 can interact with other transcription factors, including heat shock factor (Hsf1; [25]) to provide additional options for AO system regulation. As mentioned above, the Nrf2 stress pathway intimately communicates with mitochondria to maintain cellular homeostasis during oxidative stress [12].

Carnitine and Nrf2 regulation: The aforementioned evidences indicate that main protective effects of LC and ALC were associated with preservation or increased activity of antioxidant enzymes and GSH in various tissues affected by stress conditions. The mechanisms involved in the regulation of antioxidant enzymes by LC in vivo have not been precisely determined yet. However, it seems likely that transcription factors, including Nrf2, are involved in this regulation. First, it was shown in vitro that treatment of astrocytes with ALC (30-100 μM) induces HO-1 in a dose- and time-dependent manner

and that this effect was associated with up-regulation of HSP60 as well as high expression of the redox-sensitive transcription factor Nrf2 in the nuclear fraction of treated cells [26]. Adipocytes cultured in the presence of LA and/or ALC (0.1, 1 and 10 μM) for 24 h were characterised by increased expression of Nrf2 [27]. However, the treatments with LA or ALC alone at the same concentrations showed little effect on mitochondrial function and Nrf2 expression. Nrf2 regulated augmented antioxidant response on administration of ALC was shown to be a crucial factor in ameliorating hypoxia-induced neurodegeneration and memory impairment [28]. Indeed, a decrease in free radical generation, lipid peroxidation and protein oxidation was also observed along with a concomitant increase in thioredoxin and reduced glutathione levels on administration of ALC during exposure to hypobaric hypoxia. It was also demonstrated that administration of ALC to hypoxic rats effectively protected hippocampal neurons from mitochondrial dysfunction, excitotoxicity, and neurodegeneration [29]. Furthermore, ALC caused increased expression of Nrf2 in the nuclear fraction of rats with a concomitant decrease in expression of the protein in the cytosolic fraction. In addition, ALC administration resulted in PPAR-γ coactivator-1α and nuclear respiratory factor-1-induced mitochondrial biogenesis, the expression of which was regulated by an extracellular-related kinase-nuclear factor erythroid 2-related factor 2 (ERK-Nrf2)-mediated mechanism. Indeed, ALC-administered hypoxic rats showed increased DNA-binding ability of Nrf2 along with upregulation of Nrf1. The authors provided evidence for Nrf2-mediated regulation of mitochondrial biogenesis through Nrf1 following ALC supplementation [29]. Administration of LC to L-NAME-induced hypertensive rats prevented decrease in Nrf2 expression in the renal cortex [30]. Indeed, it was shown that LC can also significantly protect ischemia-reperfusion injury due to the overexpression of HO-1 induced by activated Keapl-Nrf2-ARE signaling pathway [31]. Furthermore, ALC administration to human lens epithelial cells treated with homocysteine, restored (increased) the levels of antioxidant proteins, including SOD, GSH-Px, Catalase, Nrf2, Keap1 and GSH [32]. Indeed, the aforementioned data clearly indicate modulating effects of LC and its derivatives on the Nrf2 system in various tissues.

Transcription factor NF-κB

The nuclear factor-kappa B (NF-κB) is an inducible transcription factor that regulates many cellular processes including immunity and inflammation. NF-κB consists of a group of five related proteins that are capable of binding to DNA. This transcription factor is activated by a wide range of stimuli including oxidative stress. It has been shown that NF-κB regulates the transcription of many different genes, including pro-inflammatory cytokines and leukocyte adhesion molecules, acute phase proteins and anti-microbial peptides [33-35]. There are some similarities in regulation of Nrf2 and NF-κB. For example, in physiological conditions, NF-κB is found in cytoplasm in an inactive state associated with the inhibitory IκB proteins preventing its binding to target sites. It has been proven that activation of NF-κB is an effective mechanism of host defense against infection and stress [36]. As a result of action of cytokines and other stressors, IκB proteins are rapidly phosphorylated by IκB kinase on specific serine residues, with following ubiquitination, and degradation by the 26S proteasome. The following release of NF-κB and its translocation to the nucleus is responsible for the transcription of target genes,

responsible for cell survival and involved with inflammation, immunity, apoptosis, cell proliferation and differentiation [37]. NF-κB transcription factors, such as p65, can combine to form hetero- and homo-dimers of different composition, providing a tool for effective regulation of different sets of gene targets [38]. There is a range of additional stimuli implicated into the NF-κB activation including, cell-surface receptors, inhibitory κB kinases, IB proteins, and factors that are involved in the posttranslational modification of the Rel proteins, etc. [33-37]. Accumulating evidence indicates that there is a complex interplay/crosstalk between Nrf2 and NF-κB pathways. For example, several Nrf2 activators can inhibit NF-κB pathway. NF-κB may also directly antagonize the transcriptional activity of Nrf2 (for review see [33]). In recent years, several compounds, including LC, have been shown to have inhibitory activities against multiple components of NF-κB activation pathway.

Carnitine and NF-κB regulation: Initially, it was shown that ALC selectively induces the expression of metabotropic glutamate receptor 2 by acting as a donor of acetyl groups, hyperacetylating p65/RelA and thus changing the activity of the NF-κB family of transcription factors [39]. Indeed, the *in vitro* proneurogenic effects of ALC appear to be mediated by affecting the NF-kB pathway and in particular by p65 acetylation, and subsequent NF-κB-mediated upregulation of metabotropic glutamate receptor 2 (mGlu2) expression [40]. *In vivo*, PLC treatment for 15 days after injury resulted in a reduction of relative rat aortic intimal volume, an increase of apoptosis, Bax up-regulation without changing the Bcl-2 level, and a reduction of NF-κB [41]. The authors also showed that the PLC-induced attenuation of NF-κB activity in intimal cells was due to the increase of IκB-α bioavailability, as the result of a parallel induction of IκB-α synthesis and reduction of phosphorylation and degradation. LC (8.3-13.1 mM) was found to significantly inhibit LPS-induced transactivation of NF-kB in LPS-stimulated macrophage cells [42]. Recently, it has been confirmed that the molecular regulation of antioxidant enzymes through an inhibition of the renin-angiotensin system and a modulation of the NF-κB/IκB system seems to be responsible for the antioxidant effect of carnitine [43]. A decrease in the expression of transcription factors Nrf2 and PPARα, together with an increase in NF-κB expression, was observed in the renal cortex of L-NAME-induced hypertensive rats compared with control rats (0.3-, 0.8-, and 13-fold, respectively). The simultaneous administration of LC attenuated these alterations, reaching values similar to those found in control rats [30,44,45]. Recently it has been shown that LC reduces NF-κB transactivational activity and then the production of TNFα, ICAM1, and MCP-1 in carboplatin-treated renal tubular cells [46]. Therefore inhibitory effects of carnitine on the NF-κB activated by various stress factors could be an important protective mechanism of the antioxidant defences in the body. It has also been reported that LC can activate another transcription factor, namely the peroxisome proliferator activated receptor alpha (PPARα).

Transcription factors PPARs

The peroxisome proliferator-activated receptors (PPARs) are a group of three nuclear receptor isoforms, PPARγ, PPARα, and PPARδ/β, identified in the 1990s in rodents and named after their property of peroxisome proliferation [47]. PPARs are ligand-regulated transcription factors that control gene expression by

binding to specific response elements (PPREs) within promoters and they affect various important cellular events including proliferation, differentiation, and apoptosis [48]. PPARs are shown to form a heterodimer with retinoid-X receptor (RXR) and bind a peroxisome proliferator response element (PPRE) on target genes [49]. It is proven that PPARs control expression of various genes that are crucial for lipid and carbohydrate metabolism being 'master' transcriptional regulators of nutrient metabolism and energy homeostasis that modulate the expression of unique constellations of genes [50]. In particular, PPARγ is considered to be the master transcription factor for adipogenesis, while PPARα mainly distributes in the tissue with a high efficiency of mitochondrial fatty acid oxidation, which highly expresses in the liver whereas PPARβ/δ expression is found to be very high in the small intestine and keratinocyte [51]. It seems likely that expression levels of PPARs are subject to regulation by diets and nutrient status in a tissue-dependent manner and the activities of PPARα and PPAR (gamma) can be regulated by phosphorylation [52]. It is also known that the three PPAR members share a high degree of homology however; they differ in tissue distribution, ligand specificity, and physiological roles [48]. In fact, all three PPARs play essential roles in lipid and fatty acid metabolism by directly binding to and modulating genes involved in fat metabolism [53]. Recently, a considerable number of papers have reviewed PPARs importance in regulation of various physiological and biochemical processes within the body [47,50,51,54-57] and the evolutionary pattern and regulation characteristics of PPARs have been analysed [58]. In particular, PPARα is activated by adiponectin and could inhibit NF-κB pathway, while PPAR (gamma) enhances insulin action, FFA oxidation, adiponectin secretion, and inhibits secretion of proinflammatory cytokines [57]. It seems likely that PPAR signaling is a part of the body's antioxidant system playing an important role in various stress conditions. In fact, the antioxidant effect of PPARα has been shown and PPAR-responsive elements (PPREs) have been identified in the promoter regions of several antioxidant genes, including catalase and Cu^{2+}/Zn^{2+}-SOD. Therefore, PPARα can bind to PPREs to promote the expression of antioxidants to inhibit oxidative stress [59-62] having a regulatory effect over the production of oxidative, proinflammatory and profibrotic mediators [63]. Furthermore, induction of PPARα by PPARα agonist WY14643 treatment ameliorated the oxidative stress and severity of liver injury and restored expression of genes altered by ethanol treatment [64].

A synergistic relationship between PPAR-signaling and the HO-system exists related to the regulation of various physiological functions. For example, PPARs suppress inflammation/oxidative stress and attenuate excessive immune responses, while agonists of PPARγ and PPARα have been shown to upregulate the HO-system. At the same time, the HO-system can enhance PPARα, and potentiates the expression and activity of PPARγ. Similar to PPARs, the HO-system has been shown to suppress inflammation/oxidative stress and modulate immune response [56].

Carnitine and PPARs regulation: The experimental evidence is accumulating from current studies in rats, mice and pigs to establish an essential role for PPARα in the regulation of carnitine homeostasis [65]. Indeed, an essential role for PPARα in the regulation of carnitine uptake and carnitine biosynthesis in rodents and pigs has been clearly established. For example, genes encoding proteins involved

in carnitine uptake and carnitine biosynthesis are transcriptionally regulated by PPARα [65]. On the other hand, molecular mechanisms of carnitine action, including its antioxidant functions, can also be mediated via PPARs. In fact, PPARα protein levels in the nucleus of murine liver cells increase constantly after LC supplementation and it was shown that transcription levels of the PPAR-binding protein (PPARbp) are also inducible by LC [66]. Indeed, the activation of PPARα is considered to play an important role in inhibiting the NF-κB-induced expression of proinflammatory mediators, including vascular cell adhesion molecule-1, interleukin (IL)-6, endothelin-1, and tissue factor in various cells [46,67]. For example, in renal tubular cells an anti-apoptotic effect of LC through PGI2-mediated PPAR-α activation has been reported. In fact, it was found that in NRK-52E cells LC increased PPARα activity more than 5-fold. These results reveal the crucial role of PPARα activation in the LC protective function on gentamicin-induced apoptosis in NRK-52E cells [68]. In fact, LC prevents carboplatin-mediated apoptosis through AMPK-mediated PPARα activation [46]. LC ameliorates colonic cancer cachexia in mice by regulating the expressions of PPARα and PPARγ. Indeed, LC supplementation significantly decreased expression of proinflammatory mediators, namely TNF-α and IL-6, and increased mRNA and protein expressions of PPARα and PPARγ in mice [69]. Carnitine also regulates myocardial metabolism by increasing expression of PPARα in alcoholic cardiomyopathy [70]. As mentioned above, a decrease in the expression of PPARα was observed in the renal cortex of L-NAME-induced hypertensive rats and the simultaneous administration of LC attenuated these alterations, reaching values similar to those found in control rats [30,44,45]. From *in vivo* and *in vitro* studies it is obvious that protective effects of LC against hypertension-associated renal fibrosis occur in a PPAR-γ-dependent manner [45]. The authors suggested that the beneficial effect of LC supplementation was associated with upregulation of both antioxidant enzymes and eNOS, and with a downregulation of both NADPH oxidase and RAS components. It seems likely that PPARα plays an important role in LC anti-apoptotic effect in renal tubular cells [71]. It is important to emphasize that LC not only positively regulated the pathways involved in oxidative stress defence but also improved AKT activation and downstream cellular signaling pathways involved in skeletal muscle atrophy process prevention [72]. Clearly effect of LC on PPARs was well demonstrated in various model system and need further investigation.

Effect of Carnitine on Vitagene Network

Considering molecular mechanisms of antioxidant protective action of carnitine it is necessary to consider its possible involvement in vitagene regulation. Indeed, it has been suggested that carnitine can affect signaling pathways that result in activation of vitagene network encoding survival proteins and affecting redox-sensitive intracellular pathways [73]. The term "vitagene" was introduced in 1998 by Rattan and later vitagene concept has been further developed and modified by Calabrese and colleagues [73-89] (Table 1). According to Calabrese et al. the term vitagenes refers to a group of genes that are strictly involved in maintenance and preservation of cellular homeostasis during stress conditions and the vitagene family includes heat shock proteins (HSPs), heme oxygenase-1 (HSP32, HO-1), HSP60 and HSP70, the thioredoxins (Trx)/thioredoxin reductase (TrxR) system and sirtuins [75,85]. The list of potential candidates to

Table 1: Major components of the vitagene network (adapted from [2,74,75])

Molecular level	Cellular level
AO defense	Cell proliferation
DNA-repair systems	Cell differentiation
Transfer of genetic information	Stability of cell membrane
Stress protein synthesis	Stability of intracellular milieu
Proteosomal function	Macromolecular turnover

Tissue and organ level	Physiological and redox control level
Neutralization and removing toxic chemicals	Stress response
Tissue regeneration and wound healing	Hormonal response
Tumor suppression	Immune response
Cell death and cell replacement	Thermoregulation
	Neuronal response

vitagene family is growing. In particular, special attention should be paid to SOD, a major inducible enzyme of the first level of antioxidant defence, which can meet selecting criteria to be included into the vitagene family [90]. The products of the vitagenes are responsible for detecting and controlling diverse forms of stress and cell injuries. The molecular mechanisms of the vitagene network operation have been reviewed in recently published comprehensive reviews [84-88] proving an essential regulatory role of the vitagene network in cell and whole organism adaptation to various stresses.

HO-1 and other HSPs

First, it was shown *in vitro* that ALC (30-100 µM) induces vitagene HO-1 in astrocytes in a dose- and time-dependent manner. This effect was associated with up-regulation of another vitagene HSP60 [91]. Similarly, ALC (150 mg/kg b.w orally for 4 months) induced vitagenes HO-1, HSP70 and SOD-2 in senescent rats. This protective effect of ALC was associated with other changes: upregulation of GSH levels, prevention of age-related changes in mitochondrial respiratory chain complex expression, and decrease in protein carbonyls and HNE formation [92]. In an *in vitro* study with human endothelial cells in culture carnitine and its acyl derivatives (at 0.5-2 mM) were shown to increase gene and protein expression of HO-1 [93]. Similarly, in humans and in an animal model it was shown that carnitine-mediated improvement in response to erythropoietin involves induction of HO-1 [94]. ALC 100 µM was also effective in primary cortical neuronal cultures: significantly attenuating amyloid-beta peptide 1-42-induced cytotoxicity, protein oxidation, lipid peroxidation, and apoptosis in a dose-dependent manner by upregulation of HSPs [95]. It seems likely that ALC exerted protective effects against oxidative stress in part by up-regulating the levels of GSH and HSPs. Indeed, LC treatment can increase level of HO-1 in the retinal ganglion cells [96]. It was shown that LC (50 µM, 100 µM and 200 µM) had protective effects on high glucose-induced oxidative stress in the retinal ganglion cells (RGCs). Indeed, in high glucose stimulated RGCs, LC treatment was associated with an increased level of Nrf2, HO-1 and γ-glutamyl cysteine synthetase [96]. Furthermore, ALC administration to human lens epithelial cells treated with homocysteine had a protective effect indicative by

restored (increased) levels of antioxidant proteins, including SOD, GSH-Px, Catalase, Nrf2, Keap1 and GSH [32]. Therefore, LC and its derivatives can perform their antioxidant function via activating HO-1. Indeed, HO induction occurs together with the induction of other heat shock proteins during various stressful conditions. Particularly, manipulation of endogenous cellular defence mechanisms, via the heat shock response, through nutritional antioxidants, including carnitine, may represent an innovative approach to therapeutic intervention in diseases [89] and protection against stresses. Indeed, by maintaining or recovering the activity of vitagenes, it is possible to improve adaptive ability of animals/poultry to withstand various stresses.

Sirtuins

It seems likely that carnitine can affect sirtuins, another vitagene playing an important role in cell adaptation to various stresses. In fact, both oxidative stress and mitochondrial damage are associated with reduced levels of renal sirtuin 3. Therefore, as expected, treatment with ALC restored SIRT3 expression and activity, improved renal function, and decreased tubular injury in mice [97]. It has been shown that ALC and sirtuins together affect mitochondria acetylation/deacetylation and thereby have the potential to regulate the cellular redox state, energy homeostasis and cell adaptation to stress [98]. From the aforementioned data it is clear that carnitine can be considered as an important regulator of the vitagene network.

Sparing Effects of Carnitine on Vitamin E

It is well known that vitamin E is main chain-breaking antioxidant in biological membranes having a unique role in the antioxidant systems [99,100]. In particular, vitamin E recycling mechanisms are considered to be the most important part of vitamin E efficacy in antioxidant defences. Indeed, when all essential elements of vitamin E recycling are present together with other antioxidant mechanisms, even low vitamin E levels in membranes, for example, in brain, can be sufficient to effectively protect the tissue against lipid peroxidation [99-101]. It seems likely that as a part of the antioxidant systems carnitine can have a sparing effect on vitamin E absorption and assimilation. For example, dietary LC (150 mg/kg diet) increased the rates and amounts of lymphatic absorption of α-tocopherol and fat in ovariectomized rats [102] and enhanced liver α-tocopherol in aging ovariectomized rats [103]. Similarly, carnitine dietary supplementation decreased lipid peroxidation and promotes increased concentrations of retinol and α-tocopherol in free-living women [104]. Furthermore, administration of LC (1.5 g/L with drinking water) to rats intoxicated with ethanol significantly decreased lipids and proteins oxidation in the serum and liver and the level of vitamin E was increased by about 20% in the liver and blood serum in comparison to the ethanol group [105]. In the irradiated rats treated with LC 1.5 mg/kg b.w, i.p. concentrations of vitamins E were higher than in those rats that were only exposed to 2.45-GHz radiation [106]. Furthermore, metabolomics analysis shows that α-tocopherol deficiency in rats was associated with a compensatory increase in carnitine content in the liver [107]. Therefore, molecular mechanisms of carnitine-vitamin E interactions need further investigation, but the effect of such interactions on the total antioxidant systems of the body could be quite significant.

Carnitine as a Part of Antioxidant Mixtures

Based on the concept of integrated antioxidant systems in the body it is obvious that dietary supplementation of synergistic mixtures of various antioxidants could have higher protective effects in comparison with individual antioxidants, including carnitine. Indeed, it is the case in biological systems. For example, LC and vitamin E in combination are shown to be effective in ameliorating ochratoxin A-altered hematological and serum biochemical parameters in White Leghorn cockerels [108]. A combination of LC and vitamin C was shown to decrease the risk of ischemia-induced necrosis in damaged tissues in rats [109]. Similarly, supplementation of vitamin E, vitamin C, and LC in combination can attenuate the oxidative stress associated with intermittent hypobaric hypoxia in rats [110]. There are a number of studies showing antioxidant protective effects of carnitine in combination with another mitochondria-related antioxidant, namely lipoic acid [111-119]. Similarly, a more complex antioxidant mixture, containing CoQ, LC, α-tocopherol and selenium was effective in decreasing DNA damage in the liver of fumonisin B1-treated rats [120]. Furthermore, a synergistic combination of ALC, folate and vitamin E provided a protection against oxidative stress resulting from exposure of human neuroblastoma cells to amyloid-beta [121]. In this system, vitamin E prevents *de novo* membrane oxidative damage, folate maintains levels of the endogenous antioxidant GSH and ALC prevents A-beta-induced mitochondrial damage and ATP depletion providing superior protection to that derived from each agent alone [121]. Supplementation of pregnant and lactating sow diet with carnitine-containing bioactive substances (a blend of flax seed, rapeseed, linden inflorescence, taurine, LC and tocopherol acetate) improved maturation of the small intestinal epithelium in their offspring during the early postnatal period [122]. Recently, it has been demonstrated that ALC, L-α-lipoic acid and silymarin had similar antioxidant effects in cisplatin-induced myocardial injury [123]. It would be advisable to assess antioxidant effects of a combination of carnitine and silymarin taking into account that both are considered to be hepatoprotectors and both are characterised by antioxidant properties [90]. In fact, it is proven that the therapeutic effect of silymarin combined with LC on non-alcoholic fatty liver disease in patients was higher than in silymarin used alone [124].

From the data presented above it is clear that the development of carnitine-containing antioxidant mixtures could be considered as an important step in stress prevention and treatment of stressed livestock animals.

Specific Protective Effects of Carnitine in Poultry Production

Based on the aforementioned data it is clear that protective effects of carnitine and its derivatives are most pronounced in various stress conditions. Indeed, by decreasing negative consequences of stresses carnitine can improve productive and reproductive performance and general health of growing chickens, parent stock, and commercial layers [125,126].

Stresses in poultry production

From a physiological point of view stress is related to deviation from optimal internal and external conditions causing disturbances in homeostasis. In poultry and pig industry, there are three major types of stress: environmental, nutritional, and internal stresses [127]. Environmental stresses started from the moment when egg is laid, since temperature variation could cause embryo to start developing (high environmental temperature) or die (low temperature or fast temperature changes). Furthermore, hatchability of fertile eggs declines with length of storage and there is increase in percentages of early and late embryonic mortality with length of storage period [128,129] and most likely this could affect epigenetic mechanisms determining chicken growth and development in later life. Additional time in hatchery during hatching is also a stress causing detrimental changes in antioxidant defences of the chick [130]. High environmental temperature is shown to be one of the most serious factors adversely affecting the laying performance in poultry. Egg production [131,132], egg weight [132-135], eggshell thickness [131,135-137], eggshell percentage [135], eggshell density [131], and eggshell breakage [136] were negatively affected by high ambient temperature stress. Elevated temperatures are also shown to increase mortality in both layers [133] and broilers [138]. Furthermore, the gastrointestinal tract is particularly sensitive to various stressors, which can cause a variety of changes, including alteration of the normal, protective microbiota [139] and decreased integrity of the intestinal epithelium [138]. Furthermore, heat stress is proven to inhibit the activity of digestive enzymes and reduce absorption and immune functions of intestinal mucosa [140]. The calbindin concentration was prominently decreased in ileum, cecum, colon, and eggshell gland under heat stress conditions which could be related to the deterioration of eggshell quality characteristics under heat stress conditions [135]. Broilers subjected to the heat stress were found to be characterised by reduced average daily gain and feed intake; lower viable counts of *Lactobacillus* and *Bifidobacterium* and increased viable counts of coliforms and *Clostridium* in small intestinal contents; shorter jejunal villus height, deeper crypt depth, and lower ratio of villus height to crypt depth [141].

The chick placement is also stressful time and the first 24 h of the chick's life are the most important [142]. It is believed that a chick should have an access to the feed and water as soon as possible after hatching to stimulate the development of the digestive and immune systems. It is important to mention that exposure to some stressors early in life can enhance the chicks' ability to cope with the same or with different stressors later. Indeed, compensatory responses occurred as the result of short-term exposure to stressors [143].

The increased lipid peroxidation and reduced activities of antioxidant enzymes in healthy chickens reared under unfavourable conditions including increased air temperature and humidity, high ammonia concentrations, and reduced light intensity were related to an induced oxidative stress [144]. It seems likely that vaccinations also cause substantial stress. Furthermore, it is generally assumed by immunologists that providing immunological defences is costly in terms of necessitating trade-offs with other nutrient-demanding processes such as growth, reproduction, and thermoregulation [145]. In particular, lipopolysaccharide injection decreased feed intake and body weight gain [146] and reduced ileal protein digestibility [147]. It is important to take into account that efficacy of vaccination is very much dependent on the immunocompetence of the birds, which could be compromised in stress conditions [99] and there is a range of immunosuppressive diseases in poultry, including bursal

disease, infectious chicken anemia and Marek's disease [148,149]. High stocking densities have been shown to be another stressful condition [150] causing decreased performance, increased mortality and prevalence of leg weakness and affecting the carcass quality of broiler chickens [151]. Transferring chickens from rearing to breeder houses is always associated with increased stress and sometimes causing feather picking and cannibalism [152]. The biggest stress for commercial layers and breeders comes at the peak of egg production. Indeed, major compounds of the egg yolk are synthesised in the liver, which is working to its maximum ability and any stress can cause a drop in egg production which very often is not coming up after the stress is removed. It is well appreciated in poultry industry that egg shell quality during the second part of egg laying is a problem, especially when layer age is past 80 weeks [153]. Indeed, most losses are related to the poor shell quality of eggs produced at the end of the production cycle. For example, Grobas et al. found that the percentage of broken eggs from Brown egg-laying hens on the farm increased from 0.43% at 22 weeks to 1.81% at 74 weeks of age [154].

Microbial and virus challenges are considered to be the main internal stresses causing detrimental consequences for productive and reproductive parameters of birds [99]. Furthermore, mycotoxins are considered to be among major feed-related stressors in poultry production [155-158] and they can cause oxidative stress and immunosuppression [159-166]. In general, all the aforementioned stresses suppress reproductive performance of breeders including reduced fertility and hatchability. Furthermore, stresses are associated with impaired feed conversion, reduced average daily weight gain and increased mortality in growing birds. The immune system is most likely to be the most sensitive to various stresses [127,149,159,167]. In fact, stress-related dysfunction of the immune system weakens natural resistance to diseases [168] and reduces efficacy of vaccinations [169] leading to significant losses in profits.

It is generally accepted that increased free radical production is the major molecular mechanism of the negative consequences of various stresses in human life and animal/poultry production and by decreasing ROS production or improving antioxidant protection, it is possible to decrease negative consequences of various stresses [1,2,90,99,127,170,171].

Protective effects of carnitine in stress conditions

Immunity: There is a substantial body of evidence indicating that LC dietary supplementation has immunomodulating effects on humoral and cell mediated immunity in chickens. For example, dietary LC supplementation (100 mg/kg diet) appeared to be beneficial for chickens in enhancing specific humoral responses on vaccination indicative by prevention of apoptotic death of B lymphocytes and enhanced IgG production in chickens, after both the primary and the secondary immunization [172]. In fact, a long-lasting increased IgG response due to dietary LC supplementation may be of major practical importance in the enhancement of protective immunity on vaccination. Similarly, enhanced specific antibody response to bovine serum albumin in pigeons due to LC supplementation (1g/L drinking water) was observed [173]. In fact, both BSA-specific IgG and IgM responses were enhanced by about 10% by LC supplementation. The effects of supplementing Leghorn-type chickens with dietary LC (1g/ kg diet) after hatching for 4 weeks were assessed in a 12-week study

[174]. It was concluded, that a short-term supply of dietary LC to a conventional commercial feed after hatching enhanced subsequent humoral immunity in Leghorn-type chickens. Indeed, an increased relative thymus weight and an enhanced serum primary antibody response to a mitogen in LC-fed birds were detected. Furthermore, LC in the diet of broiler chickens (100 mg/kg diet) can enhance or advance the acute phase protein response [175]. Indeed, after a LPS challenge of male broiler chickens the elevations in circulating hemopexin and alpha-1 acid glycoprotein levels were more pronounced in the LC supplemented chickens than in control birds. In ascites-susceptible broilers serum IgG content was improved by LC supplementation (75-100 mg/kg diet) [176]. Adding LC (300 mg/kg diet) into the chicken diet had a significant effect on Newcastle disease antibody titre at day 32 [177]. It seems likely that a combination of LC and methionine can also improve humoral immunity. Indeed, highest levels of IgG and WBC were found in birds fed 130% NRC methionine + 150 mg/kg LC [178]. Supplementation of LC (200 mg/kg) to broiler chickens reared at high altitude increased plasma nitric oxide and immune responsiveness, which manifested in an increased toe-web thickness index measured at 24 h following the injection of phytohemagglutinin, an *in vivo* indicator of cell-mediated immune responses [179]. Furthermore, supplemental LC (100-400 mg/kg diet) enhanced the humoral and cell mediated immune responses in Japanese quail as evidenced with better antibody titres against Newcastle disease virus and greater wing web swelling in response to PHA-L injection, respectively [180]. Indeed, carnitine can have beneficial impact on chicken immunity participating in preventing infection in commercial poultry production.

Immunomodulating properties of carnitine were also shown in farm and laboratory animals. For example, in pigs, white blood cell and lymphocyte concentrations were increased by LC dietary supplementation (250 mg/kg diet) for 10-weeks [181]. There is also evidence that LC can improve innate immunity by modulating macrophage and neutrophil activities. For example, treatment with LC (300 mg/kg b.w) significantly improved neutrophil functions, delayed-type hypersensitivity responses and the concentrations of immunoglobulins A and G in aged rats [182]. LC is also capable of restoring the age-related changes of neutrophil functions. Indeed, the neutrophils of aged rats exhibited an increase in superoxide anion production and decline in phagocytosis and chemotaxis when compared with that in young rat neutrophils. Superoxide anion production in aged rats was significantly decreased by LC treatment (50 mg/kg b.w for 30 days) which was accompanied with a significant enhancement of chemotactic and phagocytic activities which were restored to control levels [183,184]. It has been shown that LC restored lymphocyte proliferative response and the lytic activity of macrophages in aged rats [185-187]. In cultured mouse hybridoma cells LC is reported to stimulate growth and antibody production [188], while in leukemic cells isovaleryl carnitine improved phagocytosis and cell killing activity [189].

Carnitine was shown to regulate immune response in various inflammation-related diseases in animal models and in humans. For example, in rodents, treatment with LC (50-100 mg/kg body weight) markedly suppressed the LPS-induced cytokine production, improving their survival during cachexia and septic shock [190,191]. Moreover LC (200 mg/kg b.w, i.p.) was reported to improve immune

responses in mice exposed to low frequency high intensity magnetic field [192]. *In vivo*, protection from trinitrobenzene sulphonic acid colitis was observed in LC-treated mice and was attributed to the abrogation of both innate and adaptive immune responses [193]. Indeed, LC has been shown to reduce CD4+ and CD8+ T cell numbers and IL-2 production in splenocytes isolated from LC-treated mice [194] and reduce TNF-α production in *Staphylococcus aureus*-stimulated human polymorphonuclear cells [195]. Decreased serum TNFα levels have been reported after LC supplementation in surgical patients and AIDS patients [196,197]. LC administration was shown to ameliorate effects of LPS on cellular and humoral immunity in testis through reduction of IL-2 and by buffering the oxidative stress-induced damage [198].

It seems likely that an anti-apoptotic action of carnitine is of great importance in its immunomodulating properties. For example, it was shown that LC inhibited apoptosis of white blood cells [199] and CD4+ and CD8+ cells [197,200]. Similarly, supplementation with a carnitine-containing formula (alpha-tocopherol, alpha-lipoic acid, coenzyme Q (10), carnitine, and selenomethionine) to healthy individuals was shown to modulate the process of apoptosis under in vivo conditions [201]. Interestingly, LC and its derivatives have been shown to reduce apoptosis through the mitochondrial pathway [202,203] and this appears to be linked with downregulating the transduction of the pro-apoptotic Fas signal and suppressing the generation of ceramide, an important endogenous mediator of apoptosis [204]. This anti-apoptotic effect of carnitine has been observed in different cells and organelles, including neurons [205], cardiomyocytes [206], hepatocytes [207], bone marrow cells [208], neuroblastoma cell line [209], retinal ganglion cells [210], renal tubular cells [46], embryonic neural stem cells [211], spinal cord and mitochondria [212]. Therefore, LC-mediated cytoprotection and immunomodulating properties are due, in part, to inhibition of the mitochondrial apoptotic pathway [213].

Gut immunity plays an important role in protection against various pathogens [171]. It seems likely that LC may re-establish equilibrium between pro-inflammatory and anti-inflammatory cytokines, reducing the former and/or increasing the latter. This action is extremely important in the gut, since the interplay between both innate and adaptive immune responses is crucial to perpetuate inflammation in the gut in various stress conditions. Indeed, LC can suppress DC and macrophage co-stimulatory molecule expression dose-dependently [193]. Therefore, there is a therapeutic potential of LC in treating the acute and chronic aspects of intestinal inflammation. It was shown that carnitine deficiency resulted in the hyperactivation of CD4+ T cells and enhanced production of the classical Th1 cytokine, IFN-γ [193] and leads to increased apoptosis of enterocytes, villous atrophy, inflammation and gut injury [214]. Similarly, mice deficient in the carnitine transporter, OCTN2, develop spontaneous atrophy of intestinal epithelial cells and colonic inflammation [215]. In contrast, LC treatment significantly inhibited both APC and CD4+ T cell function, as assessed by the expression of classical activation markers, proliferation and cytokine production [193]. Indeed, LC has a protective effect on the intestinal mucosa by preventing ROS production [171].

However, the role of LC on immunological functions in various stress conditions still remains to be explored and the precise mechanisms of immunomodulating action of LC remain elusive. However, there are a number of potential mechanisms, which may be related to this effect. It is well recognised that sophisticated antioxidant defences directly and indirectly protect the host against the damaging effects of cytokines and oxidants. In particular, indirect protection is afforded by antioxidants, which reduce activation of NF-κB, thereby preventing up-regulation of cytokine production by oxidants. On the other hand, cytokines increase both oxidant production and antioxidant defences, thus minimising damage to the host. Antioxidants prevent oxidative stress-induced damage to immune cells. It is necessary to take into account that cellular integrity is very important for receiving, and responding to the messages needed to coordinate an immune response. The immune system generates ROS as part of its defence function and these ROS are an important weapon to kill pathogens. However, chronic overproduction of ROS can cause damage to immune cells and compromise their function [216]. In fact immune cells are rich in PUFAs which are very susceptible to free radical attack. It is well recognised that many immunological functions are membrane-dependent. These are antigen recognition, receptor expression, secretion of antibodies and cytokines, lymphocyte transformation, and contact cell lysis [216]. In particular, the receptors are important for antigen recognition and the secretion of various chemical mediators such as interferon, tumor necrosis factor, prostaglandins and interleukins. Therefore, lipid peroxidation can change membrane structure and properties (e.g. fluidity, permeability, flexibility etc.) which would affect immune cell functions. In contrast, antioxidants are able to prevent those damaging effects of ROS and maintain immune function.

If immune system is considered as "an army" fighting against invaders (microorganisms, viruses, etc.) then one would expect them to have something like mobile phones to receive and send signals to each other. Remarkably enough, major immune cells (macrophages, neutrophils, T- and B-lymphocytes) have on their surface something like "mobile phones" called receptors. Those receptors are extremely sensitive to communicating molecules, but they are also sensitive to free radicals and can be easily damaged [99,127]. In such a situation without proper communication all those huge armies of immune cells would become useless. They also can start fighting each other as well and eventually destroying immunocompetence and causing autoimmune reactions. If immune cells are considered as "soldiers" using chemical weapon to kill enemy, than special ammunition protecting them from their own weapon would be a crucial for effective battle. In the case of immune cells such ammunition is represented by natural antioxidants. Indeed if not properly protected, macrophage functions could be compromised including initial overproduction of free radicals with consecutive damages to specific enzymatic systems resulting in decreasing efficiency of oxidative burst and apoptosis. Based on the presented model it is clear that antioxidant defence is a crucial factor of immune defence in the body. Indeed, the crucial role of various receptors in immunocompetence processes and receptor sensitivity to ROS need further investigation, since exact biological/ biochemical mechanisms by which oxidants/antioxidants regulate immunity are still ill-defined. In particular, recent data have shown that TCR-induced ROS generation may be an important regulator of T cell signal transduction and gene expression [217]. Data on

the redox dependence of signal transduction in T cells are quickly growing. Recent data suggest an underlying regulatory role for ROS in controlling the susceptibility of T cells to apoptosis [218] and innate immunity efficacy [219]. In particular, recently it has been shown that mitochondria-dependent signaling controls innate and adaptive immune responses [220]. Clearly, oxidative stress leads to accrual of damaged/misfolded proteins, lipids and causes inflammation [127].

It is important to mention that individual cells and multicellular organisms have developed intricate effective mechanisms to utilize ROS and RNS to modulate homeostasis and respond to threats. Therefore, ROS and RNS are active participants in innate and acquired immune responses. Antioxidant nutrients such as carnitine may protect against oxidant-mediated inflammation and tissue damage by virtue of their ability to upregulate the antioxidant defences and optimise redox signaling, including activation of Nrf2 and prevention of the activation of NF-kB. In fact, NF-kB is required for maximal transcription of many inflammatory cytokines and adhesion molecules [221]. Therefore, LC may have great anti-inflammatory properties via downregulation of TNF-α and inhibition of NF-κB. Thus, maintaining adequate antioxidant status may provide a useful approach in attenuating the cellular injury and dysfunction observed in some inflammatory disorders [222]. It is necessary to underline that, non-toxic concentrations of ROS and RNS play an important role in regulating the expression of genes involved in the inflammatory response and in modulating apoptosis [223]. At the same time, an immune response requires extensive communication between a wide range of cell types [224] and special cell receptors are of great importance in this communication. Therefore, protective effect of antioxidants, including carnitine, in prevention of membrane and receptor damages due to peroxidation could provide an important way of enhancing the immune system.

In addition to the aforementioned mechanisms of immunomodulating properties of carnitine evidence from both animal and human studies suggests that, at pharmacological doses, LC may mimic some of the actions of glucocorticoids, including their well-known immunomodulatory effect. Indeed, LC can activate glucocorticoid receptor-α (GRα) and, through this mechanism, regulate glucocorticoid-responsive genes, potentially sharing some of the biological and therapeutic properties of glucocorticoids [225]. In fact, LC reduced the binding capacity of GRα, induced its nuclear translocation, and stimulated its transcriptional activity. Moreover, LC suppressed TNFα and IL-12 release from human monocytes in glucocorticoid-like fashion [226]. It was suggested that LC is a "nutritional modulator" of the GR, by acting as an agonist-like compound [227]. While the above data suggest immunomodulating and anti-inflammatory roles for LC, some early studies have been reported contradictory results, in part reflecting the complexity of the immune response and great variation between experimental conditions [228-230].

Therefore, LC is shown to enhance immunocompetence of birds by improving humoral and cell-mediated immunity. Furthermore, LC can decrease negative consequences of post-vaccination stress and increase vaccination success and clearly effects of carnitine on innate and acquired immunity in avian species awaits further investigation.

Ascites: Ascites syndrome (pulmonary hypertension syndrome,

PHS) is a serious metabolic disease causing important economic losses to the poultry meat industry. It seems likely that interactions of genetic, physiological, environmental, and management factors are responsible for this syndrome [231]. Furthermore, it is proven that the elevated ROS production and compromised antioxidant defences are involved in the development of ascites [232-234]. Therefore, protective effects of nutritional antioxidants are of great importance [235,236].

In this respect, based on results showing positive effects of LC dietary supplementation on growing chickens at various temperature regimes, Buyse et al. suggested that LC is a potential agent for reducing the incidence of metabolic diseases in broiler chickens [237]. Indeed, LC (75 or 150 mg/kg) or LC+CoQ10 dietary supplementation increased SOD activity and reduce ascites mortality of broilers [238]. Similarly, supplemental LC (100 mg/kg diet) reduced plasma MDA, increased SOD, inhibited remodelling and postponed the occurrence of PHS for 1 week in cold-exposed broilers [239]. Indeed, in broilers reared under low temperature environment dietary LC supplementation (100 mg/kg) reduced organ index, enhanced antioxidative capacity of the heart (SOD and GSH-Px), and enhanced liver enzymes activity involved in tricarboxylic acid cycle, and reduced serum glucose and triglyceride [240]. Dietary LC (50-150 mg/kg diet) improves pulmonary hypertensive response in broiler chickens subjected to hypobaric hypoxia and reduces ascites mortality in broiler chickens by increased NO production, reduced MDA concentration, and reduced right ventricular hypertrophy [241]. Supplementation of LC (200 mg/kg diet) had also beneficial effects on preventing lipid peroxidation and pulmonary hypertension in broiler chickens raised at high altitude (2100 m above sea level) [179]. Furthermore, dietary LC supplementation (100 mg/kg diet) of reduced-protein diets had beneficial effects in preventing pulmonary arterial hypertension mortality mainly through enhancing blood NO concentration [242]. Clearly, LC supplementation can be used to decrease detrimental consequences of ascites.

Other commercially relevant stresses: The reduction of heat production in exercising pigeons after LC supplementation [243] could be very relevant for protective effect of carnitine in heat-stressed animals/birds. For example, LC supplementation with drinking water significantly prevented deterioration of some egg quality characteristics (relative albumen weight and height) of layers under high environmental temperature [244]. It was also shown that dietary supplemental LC (50 mg/kg diet) or LC + ascorbic acid had positive effects on body weight gain and carcass weight under high temperature conditions [245].

LC (1 g/kg diet) or its combination with vitamin E (200 mg/kg diet) ameliorated ochratoxin A-induced alterations in haematological and serum biochemical parameters [108]. LC dietary supplementation (400 mg/kg diet) also has a protective effect on lipid peroxidation and drop in performance of laying hens fed high cupper diet [246]. Carnitine can also help with stresses associated with chicken placement and their first days of life. For example, Nouboukpo et al. supplemented LC in drinking water (30-60 mg/L) to broiler chickens and observed improved growth rate for the first 7 days of rearing [247]. Indeed, recent data indicate that ALC supplementation at low levels (50 or 100 mg/kg) improved antioxidative ability (increased total

antioxidant capacity and SOD and GSH-Px activities and decreased levels of MDA in serum and liver of birds), energy metabolism, and lipid metabolism in broilers [248]. There were also synergistic effects of the combined supplementation of ALC and another antioxidant, namely lipoic acid, indicative by serum and liver SOD activities and serum glucose and TG levels [248]. Carnitine can also help with age-related stresses in poultry. For example, LC supplementation (50-500 mg/kg diet) of a practical layer diet of old (65-week-old) laying hens kept in cages for 8 weeks improved egg white quality indicative by increased Haugh units [249].

From Understanding Molecular Mechanisms of Carnitine Action to the Development of Anti-Stress Compositions

Taking into account the aforementioned data on antioxidant action of carnitine and results of our recent research [1,2,90,99,100,127,170,171] it is clear that in order to deal with commercially-relevant stresses in poultry and pig production it is necessary to improve their adaptive ability to stresses. For this purpose, it would be desirable to develop a carnitine-containing products meeting at least five important requirements [1]:

1. Vitagene activation and redox-signaling (carnitine, betaine, vitamins A, E, D, C, Se, Zn, Mn, silymarin and possibly other phytochemicals);

2. Maintenance of the vitamin E recycling system (vitamin C, Se, Vitamin B_1 and B_2);

3. Provision of nutrients required for carnitine synthesis (lysine and methionine, ascorbic acid, vitamin B_6 and niacin);

4. Supporting the liver, a main site of T-2 toxin, ochratoxins, fumonisins, and aflatoxins detoxification and gut, responsible for DON detoxification (vitamins E and C, selenium, carnitine, betaine, organic acids, methionine and lysine);

5. Design of the immunomodulating mixture (vitamins A,E, D, C, carnitine, Se, Zn and Mn).

It seems likely that there should be a range of products developed to accommodate all the aforementioned requirements. In commercial conditions inclusion of various anti-stress protective compounds into the diet of pigs and poultry is complicated and difficult to implement. Firstly, a decreased feed consumption at time of stress can be observed. Secondly, such an approach has a low flexibility, since existing feeding systems do not allow to include anything into the feed loaded into the feed storage bins (usually several tons of feed for several days feeding). Therefore, before the previous feed is consumed, it is almost impossible to add anything to the feed. However, in commercial conditions there are situations when it is necessary to supplement animals/poultry with specific additives very quickly to deal with consequences of unexpected stresses (e.g. mycotoxins in the feed, immunosuppression, high temperature, etc.). In such a case water-soluble additive supplementation via drinking system is a valuable option. In fact, modern commercial poultry and pig houses are equipped with water medication systems, which can be effectively used for the aforementioned supplementations. For example, an attempt to address the aforementioned option was implemented in a commercial product PerforMax, containing a synergistic mixture of

28 compounds, including carnitine, vitamins, minerals, betaine and amino acids and supplied via drinking water. Recently commercial options and efficacy of fighting stresses by supplying the anti-stress composition via drinking water have been reviewed [170] and prospects of its use to maintain gut health in weaned piglets and newly hatched chicks were considered [171]. Indeed, supplying the PerforMax with drinking water was shown to have protective effects in growing birds in terms of improving FCR and chicken daily weight gain [250,251] as well as in adult birds: increasing egg production, eggshell quality, fertility and hatchability [170]. Therefore, decreasing detrimental consequences of stresses help maintaining chicken health, productive and reproductive performance. Therefore, the aforementioned results are the first step to go from the development of the vitagene concept to the development of commercial products addressing stress-related issues in commercial poultry and pig production. It could well be that this idea might also be realized in human nutrition. Clearly more research is needed to understand a fundamental role of vitagenes in adaptation to various stresses and use carnitine-containing antioxidant compositions to affect the vitagene network and adaptive ability of livestock animals to stresses. Indeed, it is just a matter of time before commercial products based on the vitagene concept found their way to the shelves of heathy nutrition shops and veterinary clinics.

Conclusions

There is a growing interest in the potential uses of carnitine in medical practice and animal/poultry industry. The molecular mechanisms accounting for the positive effect of LC on farm animals and poultry are not yet fully elucidated but many protective effects of LC in various stress conditions reported in literature, have been related to its antioxidant action. Based on the analysis of the recent publications it could be concluded that antioxidant actions of carnitine are associated to much extent with its role in redox signaling in the cell. Indeed, LC is shown to upregulate Nrf2 and PPARs and downregulates NF-κB leading to anti-apoptotic and anti-inflammation actions of carnitine. Furthermore, Nrf2 mediated synthesis of antioxidant enzymes, including SOD, GSH-Px, GR, GST and GSH, in response to carnitine supplementation could be a main driving force of antioxidant action of carnitine and its derivatives. In addition, LC and its derivatives are shown to also affect vitagene networks resulting in increased adaptive ability to stresses via additional synthesis of antioxidant-related molecules, including heat shock proteins (HO-1), upregulating sirtuins, thioredoxins and SOD. It seems likely that in biological systems *in vivo* the interactions of the aforementioned mechanisms provide an important place for carnitine to be a crucial part of the integrated antioxidant systems of the animal and human body. Taking into account low carnitine content in grains and poultry and pig diet formulations with limited amounts of animal proteins, carnitine requirement and possible inadequacy in commercial poultry and pig nutrition should receive more attention. Furthermore, protective roles of carnitine in stress conditions of commercial poultry and pig production, including its immunomodulating properties, are difficult to overestimate. Therefore, a development of carnitine-containing antioxidant compositions supplying via drinking water seems to be an important way forward in decreasing the detrimental consequence of various stresses in poultry and pig production. Indeed, the first steps in these

directions have been shown to be promising.

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