Heat exposure affects jejunal tight junction remodeling independently of adenosine monophosphate-activated protein kinase in 9-day-old broiler chicks

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ABSTRACT Dysfunction of the intestinal epithelial barrier under elevated temperatures is assumed to prompt pathological conditions and to eventually impede chickens' growth, resulting in massive economic losses in broiler industries. The aims of this research were to determine the impact of acute heat stress on the intestinal tight junction network of broiler chicks (*Gallus domesticus* L.) and to elucidate whether adenosine monophosphate-activated protein kinase (AMPK) was involved in the integrated response of the broiler's gastrointestinal tract to heat stress. A total of 80 9-day-old Arbor Acres chicks were subjected to temperature treatment (thermoneutral versus heat stress) and AMPK inhibition treatment (5 mg/kg body weight intraperitoneal injection of compound C vs. sham treatment) for 72 h. In addition to monitoring growth performance, the mRNA and protein levels of key tight junction proteins, target components of the AMPK pathway, and biomarkers of intestinal inflammation and oxidative stress were assessed in the jejunum under both stressors at 24 and 72 h. An increase of the major tight junction proteins, claudin-1 and zonula occludens-1, was implemented in response to an exacerbated expression of the AMP-activated protein kinase. Heat stress did not affect zootechnical performance but was confirmed by an increased gene expression of heat shock proteins 70 and 90 as well as heat shock factor-1. In addition, hyperthermia induced significant effects on tight junction proteins, although it was independent of AMPK.

Key words: intestinal integrity, tight junction, AMP-activated protein kinase, heat stress, broiler

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INTRODUCTION

Today, heat stress remains one of the most challenging worldwide concerns affecting poultry production and results in substantial economic losses due to poor performance, mortality and poor meat quality (Humphrey, 2006; Lara and Rostagno, 2013). Broiler exposure to heat stress conditions promotes detrimental physiological disturbances ranging from intestinal dysbiosis to immune system dysregulation, impaired blood electrolyte balance, and gut barrier dysfunction (Burkholder et al., 2008; Quinteiro-Filho et al., 2010; Sohail et al., 2012). The intestinal system is predominantly responsive to hyperthermia (Lambert, 2008). Under harsh conditions, the mucosal defense of the small intestine is impeded and is unable to prevent bacterial translocation, therefore increasing the risk of infection and leading to an exacerbated inflammatory response (Liu et al., 2012). Nevertheless, little attention has been paid to the underlying gastrointestinal response under high temperatures.

The selective intestinal barrier is restricted by tight junctions, which form a multiprotein network that fastens neighboring cells together and therefore seals off the intercellular cleft (Farquhar and Palade, 1963). Tight junctions are the gatekeepers of the paracellular transport pathway, ensuring the integrity of the intestinal barrier (Mandel et al., 1993), isolating the luminal region of the intercellular space, and restricting paracellular transit. Therefore, tight junction disruption may lead to increased gut permeability and eventually to a dysregulation of the body's homeostasis.

The cytoskeletal microfilaments assemble an acto-myosin ring as a belt-like structure that encircles the epithelial cell. The contraction of the perijunctional acto-myosin ring is controlled by the myosin light chain (**MLC**) (Madara, 1987; Shen et al., 2006). The regulatory MLC phosphorylation by myosin light chain kinases (**MLCK**) enhances the myosin contractility within the ring causing a shortening and opening of the

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tight junction and subsequently an increase in paracellular permeability (Rodgers and Fanning, 2011).

Through inflammation and hypoxia, heat overload is one of many factors that compromises paracellular integrity, causing oxidative enterocyte damage (Moseley et al., 1994; Hall et al., 2001). It has been underlined that heat stress leads to the enhancement of heat shock protein (**HSP**) synthesis via the phosphorylation of heat shock factors (**HSF**) (Voellmy and Boellmann, 2007). Indeed, HSP play a role in the regulation of protein homeostasis by preventing the misfolding of proteins and ensuring the degradation of denaturated proteins due to high temperatures (Kalmar and Greensmith, 2009).

A dysfunctional, also designated "leaky," tight junction barrier enhances the mucosal translocation of noxious luminal components, which promotes the exacerbated inflammatory response and endotoxemia. In turn, this exaggerated response induces pathophysiological conditions and leaves the animal more prone to infectious diseases and organ failure (Söderholm and Perdue, 2001; Groschwitz and Hogan, 2009). Another aspect of an altered intestinal permeability may include changes in nutrient digestibility and absorption across the intestinal epithelium (Pearce et al., 2013a).

Nevertheless, relatively little is known about the influence of heat stress on tight junction integrity in broiler chickens. Song et al. (2014) noticed a decrease in occludin and zonula occludens-1 (**ZO-1**) protein abundance in the jejunal mucosa of heat-stressed chickens after 10 h of exposure. In contrast, Varasteh et al. (2015a) perceived an increase in claudin-5 and ZO-1 mRNA levels after 5 d of exposure to elevated temperatures, whereas there was no marked alteration in the occludin and claudin-1 gene expression in the jejunum.

Adenosine monophosphate-activated protein kinase (**AMPK**) is a conserved serine-threonine kinase that acts as a central metabolic checkpoint and plays a key role in whole-body energy homeostasis (Hardie, 2004, 2011). AMPK is allosterically activated through fluctuations in the adenosine monophosphate (**AMP**)/adenosine monophosphate (**ATP**) ratio and by the phosphorylation of its threonine-172 residue by upstream kinases, among which liver kinase B1 (**LKB1**) is the most recognized (Corton et al., 1994, 1995; Hawley et al., 1995). AMPK activity is triggered by physiological or pathological stimuli caused by ATP depletion, such as hypoxia, oxidative stress, heat shock, ischemia, nutrient deprivation, metabolic poisoning, muscle contraction, and exercise (Hardie, 2004). Once activated, AMPK phosphorylates its downstream targets, and the cascade is aimed at restoring cellular energy levels by switching off ATP-consuming pathways and switching on ATP-generating processes (Hardie and Carling, 1997). Indeed, AMPK is characterized by its pleiotropic ability to govern a wide range of metabolic processes reviewed by Hardie (2004, 2011), involving the lipid, cholesterol, protein, and glucose metabolisms, in specialized tissues such as the liver, pancreas, muscle, and adipocytes. Recent studies by Zhang et al. (2006) and Zheng and Cantley (2007) underscored the physiological relevance of AMPK as a key player in tight junction assembly.

Thus far, the upstream signaling pathway regulating tight junction remodeling under hyperthermia remains unresolved. Therefore, the aim of this research was to further characterize the impact of heat stress on the tight junction intestinal barrier in broiler chicks (*Gallus gallus domesticus* L.) and to assess the involvement of AMPK and its upstream kinase, LKB1, in this process.

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted with 80 1-day-old Arbor Acres male broiler chicks (*Gallus gallus domesticus* L.) from a local hatchery (Da Bao Hatchery, Tai'an, China). After a 9-d acclimation period, the experiment started and consisted of a 2-way factorial design, with 2 experimental treatments, i.e., temperature and AMPK inhibition. The trial was prepared using a randomized block design with 40 broilers assigned to the thermoneutral temperatures, while an equal number were exposed to heat stress. Compared with a temperature of 30°C for the thermoneutral room, the temperature in the heat stress room was maintained at 39°C throughout the entire experiment with a relative humidity of 60% in both rooms. For each temperature setup, half of the chicks received a single injection of compound C (5 mg/kg body weight; Sigma-Aldrich Co., St Louis, MO) as AMPK inhibitor; this compound was dissolved in warm dimethyl sulfoxide (Sigma-Aldrich Co) on the first day of experimentation (10 d old). The others were treated with an equal amount of dimethyl sulfoxide to ensure uniform treatment. Every group was subsequently organized into 4 cage replicates of 5 birds each. To measure an eventual acclimation to the induced stressors, the time course effect of the treatments was investigated with data collected at 24 and 72 h post-treatment exposure. All chicks had ad libitum access to water and standard commercial feed (starter diet: 21.5% CP; 12.81 MJ/kg ME). The diet was provided by a local feed mill (Rolin Animal Nutriment & Health Products Co. Ltd., Tai'an, China) under mash form. The light regime was set on 23-h light and one-h darkness throughout the entire experiment.

This study was approved by the ethics committee of Shandong Agricultural University and conducted in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology of China (Beijing, P. R. China).

Zootechnical Performance

The body weight per cage was recorded on the first day of the experiment, prior to the assignment of the cages to specific treatments, and 24 and 72 h

Table 1. Nucleotide sequences of primers for the gene expression analysis.

Target gene		Primer sequence 5′ → 3′ (F: forward, R: reverse)	Genebank accession number
Occludin	F	TCATCGCCTCCATCGTCTAC	NM_205128.1
	R	TCTTACTGCGCGTCTTCTGG	
Claudin-1	F	CTGATTGCTTCCAACCAG	NM_001013611
	R	CAGGTCAAACAGAGGTACAAG	
ZO-1	F	CTTCAGGTGTTTCTCTTCCTCCTC	XM_413773
	R	CTGTGGTTTCATGGCTGGATC	
AMPKα1	F	AAGGTTGGCAAGCATGAGTT	XM_424772
	R	TTCTGGGCCTGCATATAACC	
LKB1	F	ATTCCAGCCACCAGAAATTG	XM_418227
	R	CCTCATTGTAGCCATGCAGA	
iNOS	F	CCT GGA GGT CCT GGA AGA GT	NM_204961.1
	R	CCT GGG TTT CAG AAG TGGC	
HSP70	F	CGTCAGTGCTGTGGACAAGAGTA	NM_001006685
	R	CCTATCTCTGTTGGCTTCATCCT	
HSP90	F	ATGCCGGAAGCTGTGCAAACACAGGACCAA	NM_001109785.1
	R	GGAATCAGGTTAATTTTCAGGTCTTTTCCA	
HSF-1	F	CAGGGAAGCAGTTGGTTCACTACACG	L06098.1
	R	CCTTGGGTTTGGGTTGCTCAGTC	
GLUT-2	F	AGAGGAAACTGTGACCCGATGGA	NM_207178
	R	AACGAAGAGGAAGATGGCGAC	
MLCK	F	AGCTGCCAGGTGTCTCTGAT	NM_001322361.1
	R	AGTTTCCACTCGCCTCTTCA	
β-Actin	F	ATGTGGATCAGCAAGCAGGAGTA	NM_205518.1
	R	TTTATGCGCATTTATGGGTTTTGT	

AMPK: AMP-activated protein kinase; GLUT-2: glucose transporter 2; HSF: heat shock factor; HSP: heat shock protein; iNOS: inducible nitric oxide synthase; LKB1: liver kinase B1; MLCK: myosin light-chain kinase; ZO: zonula occludens.

post-treatment induction. The absolute live weight per cage was used to calculate the average relative body weight gain (**BWG**). Daily feed intake per pen was measured and the relative feed intake was subsequently calculated. The absolute FI and BWG were used to calculate the feed conversion ratio (**FCR**) per pen. Mortality was recorded by daily visual observation during the experimental period.

Sample Collection

At 24 and 72 h post-treatment exposure, 2 chicks per cage-replicate with similar weight to the cage average body weight were sacrificed by decapitation. Tissues were dissected from the mid-jejunum. The jejunum was opened lengthwise and gently flushed twice with physiological saline and the jejunum surface was scraped using a sterile glass microscope slide to collect the mucosa. The samples were snap-frozen in liquid nitrogen and stored at –80°C until further analyses.

Quantitative Reverse Transcription PCR Analysis

Total RNA was extracted from the jejunal mucosa tissues according to the Total RNA Kit II manufacturer's instructions (Omega Bio-Tek Inc. Ltd., Norcross, Georgia). RNA concentration of each sample was determined by Nanodrop technology. Reverse transcription (RT) reactions were performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany). Real-time PCR analysis was conducted using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each RT reaction served as a template in a 20 μL PCR mixture containing commercially manufactured gene-specific primers (Table 1; 300 nM; Sangon Biotech Co. Ltd., Shanghai, China) and SYBR Green master mix (Roche Applied Science). Pooled samples were produced by mixing the cDNA templates of all the experimental samples with 5-fold serial dilutions and were used to optimize qRT-PCR conditions by calibrating standard curves and calculating the PCR efficiencies. All runs were performed with a default RT-PCR protocol, with a pre-denaturation step (10 min, 95°C) followed by amplification for 40 cycles with a denaturation step (15 s, 95°C) and an annealing and extension step (1 min, 60°C). PCR products were detected by measuring SYBR Green fluorescence at the end of each cycle. For each target gene, the relative gene expression levels were calculated by the 2-$\Delta\Delta$ cycle threshold (**Ct**) method using β-actin as the housekeeping gene (Livak and Schmittgen, 2001).

Western Blot Analysis

Protein extraction was performed using the Protein Extraction Kit (Beyotime Institute of Biotechnology Co. Ltd., Jiangsu, China). The total protein concentration for each lysate was quantified using the bicinchoninic acid assaying kit (CWBioTech Co., Beijing, China), and equal protein amounts of the boiled samples (30 μg) were separated by SDS-PAGE on 8.5% polyacrylamide gels (CWBioTech Co.). The

Table 2. Effects of AMPK inhibition treatment after 24 and 72 h of stress exposure on the growth parameters (n = 4).

	AMPK inhibition treatment (24 h)		AMPK inhibition treatment (72 h)	
	Sham-treated	Compound C	Sham-treated	Compound C
Relative BWG[1] (%)	14.04 ± 0.71[a]	9.88 ± 0.85[b]	41.79 ± 2.46[a]	29.54 ± 1.76[b]
Relative FI[2] (g/100 g)	15.84 ± 0.56[a]	12.24 ± 0.83[b]	41.94 ± 1.37[a]	34.98 ± 1.71[b]
FCR	1.14 ± 0.04[b]	1.28 ± 0.09[a]	1.44 ± 0.06[b]	1.72 ± 0.08[a]

[a,b]Different superscript letters ([a, b]) denote significant differences between the experimental treatments at that time point ($P < 0.05$).

AMPK: AMP-activated protein kinase; BWG: body weight gain; FCR: feed conversion ratio; FI: feed intake.

[1]Relative BWG (%) = (BW24 or BW72 – BW0) × 100/BW0; where BW0: body weight at 0 h (average initial body weight was 42.6 g), BW24: body weight at 24 h, and BW72: body weight at 72 h.

[2]Relative FI (g/100 g) = (FI × 0.01)/BW0.

proteins were then electro-transferred onto polyvinylidene difluoride membranes (Immobilon-P membrane, Merck Millipore Corporation, Darmstadt, Germany). The blot was rinsed in Tris Buffered Saline with Tween 20 (**TBST**) buffer and then blocked in a commercial blocking buffer (Beyotime Institute of Biotechnology Co. Ltd.). Next, the membrane was incubated with primary antibody solutions [AMPKα, p-AMPKα, and LKB1; 1:1000; Cell Signaling Technology Inc., Danvers, Massachusetts, and Occludin; 1:1000; Abcam Plc., Cambridge, UK] overnight at 4°C. Membranes were subsequently probed with a β-actin antibody (1:1000, Beyotime Institute of Biotechnology Co. Ltd.) to evaluate the loading equality. After washing in TBST, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody solutions (1:1000; Beyotime Institute of Biotechnology Co. Ltd.) for 4 h at 4°C. Finally, the blots were washed in TBST and incubated with enhanced chemiluminescence detection reagent (Beyotime Institute of Biotechnology Co. Ltd.). The membranes were then scanned using Fusion Fx (Vilber Lourmat SAS., Collégien, France), and signal intensities of the target proteins were calibrated by densitometry using Fusion-Capt Advance FX7 software optical density analysis (Vilber Lourmat SAS). Protein abundance was normalized to β-actin.

Statistical Analysis

Prior to the analysis of variance, homogeneity among variances and normality among treatments were confirmed using Bartlett's and Ryan-Joiner's tests, respectively. The experimental data concerning the broiler growth performance and laboratory procedures were subjected to a GLM procedure using a hierarchical approach with the treatment duration, temperature, and AMPK inhibition treatments as fixed effects. The statistical model included the effects of temperature, AMPK inhibition, treatment duration, and their interactions as factors. When the GLM displayed significant interactions between the 2 experimental stressors, the differences among the means were evaluated by Tukey's multiple range test to assess contrasts among the 4 groups. Data analysis was performed with SAS 9.4 statistical analysis software. P-values <0.05, <0.01, and <0.001 were considered significant, highly significant, and very highly significant, respectively. Instances in which P-values < 0.1 were said to indicate trends. The resulting data are organized as tables presenting the mean ± standard error for each treatment. The size of each population used for statistical analysis is indicated in the title of the respective table.

RESULTS

Phenotypic Response to Heat Stress and AMPK Inhibition

At both time points, i.e., after 24 and 72 h of stress exposure, AMPK inhibitor injection reduced broiler chicks' performance parameters ($P < 0.05$; Table 2). Conversely, no significant difference in productivity was observed following the temperature treatment, regardless of treatment duration (Table 3). No significant interaction between the temperature and AMPK inhibition treatments was recorded for the performance parameters. No mortality occurred throughout the entire experiment.

Biomarkers Responses to Hyperthermia and AMPK Inhibition Condition

Significant interactions were observed for the inducible nitric oxide synthase (**iNOS**) and heat shock factor 1 (**HSF-1**) after 72 h ($P < 0.05$). Mucosal iNOS, HSF-1, and HSP70 and HSP90 gene expression levels were increased under hyperthermia after 72 h of stress ($P < 0.05$; Table 4). HSP90 levels were already significantly higher in heat-stressed chickens after 24 h ($P < 0.05$; Table 4). Chicks injected with compound C displayed higher levels of HSF-1 compared to the sham-treated counterparts after 24 h ($P < 0.05$; Table 4), whereas the same effect was observed for iNOS after 72 h ($P < 0.01$; Table 4). HSP70 and HSP90 jejunal mucosal levels were not affected by the AMPK inhibitor.

Table 3. Effects of temperature treatment after 24 and 72 h of stress exposure on the growth parameters (n = 4).

	Temperature treatment (24 h)		Temperature treatment (72 h)	
	Thermoneutral	Heat stress	Thermoneutral	Heat stress
Relative BWG[1] (%)	11.04 ± 1.30	12.89 ± 0.72	36.47 ± 3.59	34.87 ± 2.60
Relative FI[2] (g/100 g)	13.62 ± 1.15	14.46 ± 0.75	38.88 ± 2.50	38.04 ± 1.39
FCR	1.28 ± 0.08	1.13 ± 0.06	1.58 ± 0.10	1.58 ± 0.08

[a,b]Different superscript letters ([a, b]) denote significant differences between the experimental treatments at each time point ($P < 0.05$).

BWG: body weight gain; FCR: feed conversion ratio; FI: feed intake.

[1]Relative BWG (%) = (BW24 or BW72 − BW0) × 100/BW0; where BW0: body weight at 0 h (average initial body weight was 42.6 g), BW24: body weight at 24 h, and BW72: body weight at 72 h.

[2]Relative FI (g/100 g) = (FI × 0.01)/BW0

Table 4. Effects of temperature and AMPK inhibition treatments after 24 and 72 h of stress exposure on the immune and stress response (n = 8).

	Thermoneutral (24 h)	Heat stress (24 h)	Sham-treated (24 hs)	Compound C (24 hs)	$P_{interaction\ 24\ h}$	Thermoneutral (72 h)	Heat stress (72 h)	Sham-treated (72 h)	Compound C (72 h)	$P_{interaction\ 72\ hs}$
iNOS	1.05 ± 0.21	0.72 ± 0.06	0.85 ± 0.23	0.92 ± 0.04	NS	1.29 ± 0.32^{b}	2.99 ± 0.38^{a}	1.28 ± 0.17^{b}	3.00 ± 0.53^{a}	<0.05
HSP70	1.01 ± 0.02	1.00 ± 0.01	1.00 ± 0.01	1.01 ± 0.02	NS	0.99 ± 0.03^{Y}	1.03 ± 0.02^{X}	1.01 ± 0.02	1.01 ± 0.03	NS
HSP90	0.85 ± 0.09^{b}	1.23 ± 0.21^{a}	1.15 ± 0.17	0.89 ± 0.13	NS	0.92 ± 0.12^{b}	1.48 ± 0.20^{a}	1.21 ± 0.15	1.20 ± 0.18	NS
HSF-1	1.06 ± 0.07	0.96 ± 0.07	0.95 ± 0.06^{b}	1.10 ± 0.07^{a}	NS	0.88 ± 0.11^{b}	1.33 ± 0.17^{a}	0.97 ± 0.09	1.24 ± 0.18	<0.05

[a,b]Different superscript letters ([a, b]) denote significant differences between the experimental treatments at each time point ($P < 0.05$).
[X,Y]Superscript letters ([X, Y]) denote statistical trends between experimental treatments ($P < 0.1$).
HSF: heat shock factor; HSP: heat shock protein; iNOS: inducible nitric oxide synthase.

Response of the AMPK Signaling Pathway

For all of the targets related to the sensor signaling pathway, no significant interaction between the temperature stress and the AMPK inhibitor was observed. Regarding AMPKα1 mRNA levels at 24 h post-stress induction, neither of the induced treatments had a significant effect on the gene expression levels of the energy sensor AMPK, whereas the analysis of the second time point revealed that AMPKα1 gene expression was upregulated following compound C injection ($P < 0.05$; Table 6) but remained unchanged during temperature treatment (Table 7). With respect to AMPKα phosphorylation levels, i.e., the ratio of the phosphorylated form of AMPKα normalized to the total amount of AMPKα, the analysis of the protein content at the first time point revealed that the injected chicks demonstrated a lower phosphorylation ratio (half-fold; $P < 0.1$; Table 6) than the untreated chicks, without reaching statistical significance. In contrast, the chicks subjected to compound C tended to exhibit a higher phosphorylation ratio (1.4-fold) than the untreated broiler chicks after 72 h post-stress induction ($P < 0.1$; Table 6).

The chicks exposed to thermal stress displayed significantly higher LKB1 gene expression levels after 72 h of stress ($P < 0.01$; Table 7) than those exposed to thermoneutral temperatures. Similarly, after 24 h of stress exposure, the LKB1 protein levels of the chicks exposed to heat stress significantly increased by 2.2-fold compared with those of the chicks exposed to thermoneutral temperatures ($P < 0.01$; Table 7). LKB1 mRNA and protein levels did not significantly differ between the chicks subjected to AMPK inhibition and the untreated chicks (Table 6).

Response of the Transcellular Pathway

A significant interaction ($P < 0.001$; Table 5) was recorded between the temperature and AMPK inhibition treatment for the glucose transporter 2 (**GLUT-2**) gene expression level after 72 h of stress exposure. Chicks injected with compound C displayed significantly lower GLUT-2 mRNA level than sham-treated chicks ($P < 0.001$; Table 5). Heat-stressed chicks also exhibited a downregulated GLUT-2 gene expression in comparison with their control counterparts after 72 h ($P < 0.001$; Table 5). None of the stressors impacted on GLUT-2 after 24 h of exposure.

Response of the Paracellular Network

For all of the targets related to the intestinal network, no significant interaction between the temperature stress and the AMPK inhibitor was observed. Regardless of the treatment duration, neither the temperature (Table 7) nor AMPK inhibition treatment (Table 6) affected the mRNA levels of occludin.

Table 5. Effects of temperature and AMPK inhibition treatments after 24 and 72 h of stress exposure on glucose transporter 2 (n = 8).

	24 h			72 h	
	Sham-treated	Compound C		Sham-treated[a]	Compound C[b]
Thermoneutral	1.00 ± 0.11	1.05 ± 0.05	Thermoneutral[a]	1.00 ± 0.03	0.36 ± 0.01
Heat stress	1.02 ± 0.04	0.91 ± 0.03	Heat stress[b]	0.43 ± 0.01	0.38 ± 0.06
	$P_{Interaction}$ = 0.2124			$P_{Interaction}$ <0.0001	

[a,b]Different superscript letters ([a, b]) denote significant differences between the experimental treatments at each time point ($P < 0.05$).

Table 6. Effects of AMPK inhibition treatment after 24 and 72 h of stress exposure on the target proteins at the mRNA and protein levels (n = 8).

	AMPK inhibition treatment (24 h)			AMPK inhibition treatment (72 h)		
	Sham-treated	Compound C	$P_{interaction\ 24\ h}$	Sham-treated	Compound C	$P_{interaction\ 72\ h}$
AMPK mRNA level	1.00 ± 0.05	1.07 ± 0.023	NS	1.01 ± 0.03[b]	1.18 ± 0.05[a]	NS
AMPK phosphorylation[1]	1.00 ± 0.22[X]	0.55 ± 0.071[Y]	NS	0.74 ± 0.05[Y]	1.04 ± 0.16[X]	NS
LKB1 mRNA level	1.00 ± 0.01	0.98 ± 0.016	NS	1.03 ± 0.02	1.02 ± 0.03	NS
LKB1 protein level	1.00 ± 0.13	1.24 ± 0.238	NS	1.03 ± 0.10	0.90 ± 0.08	NS
Occludin mRNA level	1.00 ± 0.01	1.03 ± 0.012	NS	0.96 ± 0.02	0.98 ± 0.02	NS
Occludin protein level	1.00 ± 0.13	0.98 ± 0.075	NS	1.06 ± 0.13	1.13 ± 0.13	NS
Claudin-1 mRNA level	1.00 ± 0.01	1.05 ± 0.021	NS	1.03 ± 0.03[b]	1.15 ± 0.05[a]	NS
ZO-1 mRNA level	1.00 ± 0.01[b]	1.07 ± 0.028[a]	NS	1.00 ± 0.032[b]	1.18 ± 0.05[a]	NS
MLCK mRNA level	1.00 ± 0.24	0.93 ± 0.18	NS	1.05 ± 0.12[b]	2.63 ± 0.63[a]	NS

[a,b]Different superscript letters ([a, b]) denote significant differences between the experimental treatments at each time point ($P < 0.05$).
[X,Y]Superscript letters ([X, Y]) denote statistical trends between experimental treatments ($P < 0.1$).
AMPK: AMP-activated protein kinase; LKB1: liver kinase B1; MLCK: myosin light-chain kinase; ZO: zonula occludens.
[1]AMPK phosphorylation: phosphorylated form of AMPK normalized to the total amount of AMPK.

Table 7. Effects of temperature treatment after 24 and 72 h of stress exposure on the target proteins at the mRNA and protein levels (n = 8).

	Temperature treatment (24 h)			Temperature treatment (72 h)		
	Thermoneutral	Heat stress	$P_{interaction\ 24\ h}$	Thermoneutral	Heat stress	$P_{interaction\ 72\ h}$
AMPK mRNA level	1.00 ± 0.02	0.97 ± 0.01	NS	1.03 ± 0.05	1.05 ± 0.04	NS
AMPK phosphorylation[1]	1.00 ± 0.11	1.06 ± 0.32	NS	1.27 ± 0.21	1.09 ± 0.11	NS
LKB1 mRNA level	1.00 ± 0.01	0.98 ± 0.01	NS	0.98 ± 0.03[b]	1.07 ± 0.02[a]	NS
LKB1 protein level	1.00 ± 0.16[b]	2.32 ± 0.30[a]	NS	1.22 ± 0.15	1.52 ± 0.14	NS
Occludin mRNA level	1.00 ± 0.01	0.98 ± 0.01	NS	0.93 ± 0.02	0.96 ± 0.02	NS
Occludin protein level	1.00 ± 0.09[b]	1.29 ± 0.15[a]	NS	1.45 ± 0.13[X]	1.06 ± 0.14[Y]	NS
Claudin-1 mRNA level	1.00 ± 0.02[Y]	1.17 ± 0.01[X]	NS	1.01 ± 0.04	1.08 ± 0.04	NS
ZO-1 mRNA level	1.00 ± 0.02	0.98 ± 0.01	NS	1.05 ± 0.05	1.04 ± 0.04	NS
MLCK mRNA level	1.00 ± 0.16	0.93 ± 0.26	NS	1.06 ± 0.25[b]	2.62 ± 0.50[a]	NS

[a,b]Different superscript letters ([a, b]) denote significant differences between the experimental treatments at each time point ($P < 0.05$).
[X,Y]Superscript letters ([X, Y]) denote statistical trends between experimental treatments ($P < 0.1$).
AMPK: AMP-activated protein kinase; LKB1: liver kinase B1; MLCK: myosin light-chain kinase; ZO: zonula occludens.
[1]AMPK phosphorylation: phosphorylated form of AMPK normalized to the total amount of AMPK.

Occludin protein levels were significantly higher in heat-stressed chicks (1.3-fold; $P < 0.05$; Table 7) than those in the thermoneutral chicks at the first time point, whereas the abundance of occludin proteins in broiler chicks subjected to elevated temperatures tended to be lower at 72 h post-treatment induction (0.7-fold; $P < 0.1$; Table 7).

Claudin-1 mRNA levels tended to vary in response to the temperature treatment, with heat-stressed chicks exhibiting the highest mRNA levels (1.2-fold; $P < 0.1$; Table 7) after 24 h of treatment exposure. After 72 h of heat exposure, the tight junction abundance in the chicks injected with compound C was significantly higher (1.1-fold; $P < 0.05$; Table 6) than that in the sham-treated broiler chicks.

Concerning the ZO-1 gene expression, ZO-1 mRNA levels significantly varied under compound C injection at both time points. The ZO-1 protein levels of the injected chicks were significantly upregulated both at 24 h (1.1-fold; $P < 0.05$; Table 6) and 72 h (1.2-fold; $P < 0.05$; Table 6) post-stress induction.

Myosin light-chain kinase mRNA levels were significantly influenced by both treatments after 72 h, with heat-stressed chicks (2.5-fold; $P < 0.01$; Table 7) as well as the compound C-injected animals (2.5-fold; $P < 0.01$; Table 6) demonstrating higher gene expression levels.

DISCUSSION

The profound, adverse effect of compound C injection on the BWG, FI, and FCR confirmed the physiological relevance of AMPK as an energy sensor (Hardie, 2004) and its effect on the feeding behavior of broilers (Kim et al., 2004).

Although a lower BWG, reduced feed consumption, and higher FCR were originally expected due to heat stress (Lei and Slinger, 1970; Geraert et al., 1996), no effects were observed on these parameters, nor were differences observed with respect to the mortality rate. The young age of the broiler chicks probably provided plasticity to cope with the acute heat stress, without affecting zootechnical performance (Altan et al., 2000; Yahav, 2000; Yahav and McMurtry, 2001; Gu et al., 2012). However, our heat stress protocol was effective considering the marked elevation of cellular stress in the form of HSF-1, HSP70, and HSP90 thereby confirming hyperthermia. Heat shock factors are phosphorylated in response to environmental stressors and enhance the transcription of heat shock proteins considered as biomarkers of stress-induced tissue injury leading to an increased antioxidant capacity (Voellmy and Boellmann, 2007). iNOS gene expression was also upregulated after 72 h of heat stress, reflective of an important oxidative stress. Tight junction proteins' assembly is weakened due to oxidative stress that follows overheating (Leon and Helwig, 2010). Higher occludin (at the protein level) and claudin-1 (at the gene level) levels in the jejunal tissues of the chicks exposed to 24 h of heat were in accordance with numerous in vitro and in vivo trials. These previous studies demonstrated that 24 h of heat stress, or less, accounted for an increase in occludin levels in Caco-2 intestinal epithelial cells (Dokladny et al., 2006, 2008; Xiao et al., 2013), IEC-6 intestinal epithelial cells (He et al., 2016), and growing pigs (Pearce et al., 2013a). A surge in transmembrane proteins after short-term hyperthermia could reflect a defense mechanism to strengthen the tight junction network and enhance the barrier tightness to cope with the potential leakage of the paracellular seal during elevated temperatures. Nevertheless, both transmembrane protein upregulations were time dependent because claudin-1 mRNA levels were unaffected within 72 h of thermal stress, whereas the occludin protein abundance tended to be reduced in a chick's jejunum under hyperthermia at this same time point. These results are in agreement with He et al. (2016), who identified a downregulation of the occludin protein levels after 3 d of intermittent heat burden in Sprague-Dawley rats and with Varasteh et al. (2015a) who found unaffected levels of claudin-1 in chicken's jejunum after 5 d of heat stress. These findings suggest that occludin expression is a time-dependent process, with a protective increase followed by a reduction in the transmembrane protein abundance, indicating a disturbed intestinal barrier. In contrast, several studies found either downregulated or unaffected occludin levels after short-term heat stress, in a broiler's jejunum (Song et al., 2014), rat intestines (Xiao et al., 2015), and in in vitro models (Xiao et al., 2013; Varasteh et al., 2015b; He et al., 2016). A plausible explanation for these discrepancies could be the duration of the application of heat exposure, the species chosen, their age, and the temperature of the exposure. Additionally, the total tight junction levels could be compartmentalized within the cell. Indeed, tight junction proteins relocalize under stressful conditions (Turner, 2006, 2009). A separate protein extraction for both the cytoplasmic and membranous contents would permit a better understanding of the internalization and externalization of the tight junctions, and therefore provide a more accurate assessment of the heat stress-mediated effect on cytoplasmic plaques. These findings would be enhanced by an assessment of barrier integrity using chambers or in vivo tests.

The contractility of the actin cytoskeleton was compromised during heat stress with an increase in MLCK gene expression levels after 72 h. The protein kinase MLCK is activated under elevated temperatures leading to the subsequent phosphorylation of MLC which increases the contractility of the acto-myosin ring causing the tight junction opening as highlighted by Yang et al. (2007) in T84 intestinal epithelial cell and by Pearce et al. (2013a,b) in growing pigs.

Furthermore, we have reported, under heat stress, a decreased intestinal membrane GLUT2 gene expression aiding in the non-energy requiring glucose transport in heat-stressed chicks which had also been reported in pig's ileum (Pearce et al., 2013a). Therefore, not only the paracellular transport was impeded but also the transcellular pathway.

According to Kodiha et al. (2007), AMPK phosphorylation is downregulated under heat stress in HeLa and HEK293 cell cultures. Moreover, the kinase also clusters in the nuclei of both cell lines as a consequence of the environmental stress (Kodiha et al., 2007). Wang et al. (2010) strengthened these findings by postulating that the AMPK activity was inhibited in several cultured epithelia (HepG2, bEnd.3, Hepa1–6, C2C12, 293T, and MIN6) under hyperthermic conditions through the dephosphorylation of the AMPKα subunit by the protein phosphatase PP2A. Wang et al. (2010) speculated that AMPK dephosphorylation was a defense mechanism for cell survival to cope with environmental stressors. No significant variations in AMPKα1 mRNA levels or AMPKα phosphorylation status in the chick's jejunum were monitored after thermal stress exposure, which is in contrast with the in vitro tests by Wang et al. (2010).

Interestingly, LKB1 gene expression (72 h after the onset of the stressor) and protein levels (24 h after the onset of the stressor) were significantly upregulated in the chicks' jejunal tissues under elevated temperatures. Nevertheless, these findings cannot be either confirmed or refuted because the effect of LKB1 under thermal stress has not yet been investigated in vitro or in vivo.

Compound C is the most commonly used AMPK inhibitor. It alters enzyme phosphorylation levels (Kim et al., 2004; Peng et al., 2009; Elamin et al., 2013) but has no apparent impact on the gene expression of the AMPK complex (Zhou et al., 2001), as confirmed in our study at 24 h post-injection. Nevertheless, the opposite trend was perceived at 72 h post-treatment induction; chicks subjected to AMPK inhibition had higher AMPKα1 gene expression levels and AMPKα phosphorylation levels compared with their control counterparts. The inhibitory effect of compound C may have dissipated after 72 h, as a compensatory increase in both the gene expression and protein levels probably increased the overall metabolism. This pattern reflects a time-dependent effectiveness of the agent that, to our knowledge, has not been reported before.

Nevertheless, compound C induced a significant increase in HSF-1 after 24 h and in iNOS after 72 h of incubation, reflecting the oxidative stress response in the gastrointestinal tract. Previous studies using smooth muscle cells reported that AMPK is a negative regulator of MLCK, responsible for the contractility of intestinal longitudinal muscles (Horman et al., 2008; Nalli et al., 2014). It is therefore conceivable that the AMPK inhibition accounts for the rise in MLCK gene expression and the subsequent muscle hypercontractility. In addition, we highlighted a decreased intestinal membrane GLUT2 gene expression after 72 h in chicks submitted to compound C which is in line with Walker et al. (2005) findings under the AMPK activators 5-aminoimidazole-4-carboxamide riboside. This implies the remaining inhibitory effect of compound C results in a downregulation of glucose uptake by GLUT2 and therefore a hampered passive transcellular transport on the basolateral side.

ZO-1 gene expression levels were upregulated whereas occludin mRNA levels and protein abundance remained stable under AMPK inhibition in the jejunal tissues after 24 h of treatment. This finding is surprising as ZO-1 and occludin levels were hypothesized to have evolved together (Fanning et al., 1998). Subsequent analyses of the cytosolic and membranous protein fractions of each target tight junction could clarify the underlying phenomenon and might reveal a greater internalization of proteins. Only a low fraction of the proteins may be present at the cell periphery because proteins can redistribute in times of thermal stress (Turner, 2006, 2009). The emerging picture after 72 h of stress exposure is that claudin-1 and ZO-1 gene expression levels were significantly upregulated in the chick's jejunum, along with the energy sensor levels at the same time. These patterns are in agreement with Park et al. (2015), who showed that the claudin-1 and ZO-1 protein abundance increased in response to the activation of the energy sensor in Caco-2 cell monolayers. Therefore, an enhancement of the key tight junction proteins, which seal off the paracellular cleft, may strengthen the intestinal epithelial function as a protective mechanism. Indeed, ZO-1 is crucial for the polymerization of claudins and determines whether they are relocalized from the cytoplasm to the cell periphery (Umeda et al., 2006). Nevertheless, the fact that occludin mRNA levels were stable in response to the AMPK inhibitor at this time point demonstrates, once again, that the tight junction network is a complex network with multifaceted regulation. Taken together, these findings suggest the functional importance of AMPK as a credible mediator of tight junction maintenance, which differs from the suggestion by Zhang et al. (2006) and Zheng and Cantley (2007), who acknowledged that the enzyme accelerates tight junction assembly but is not required for tight junction maintenance.

Characterization of the AMPK-LKB1 signaling pathway under hyperthermia has shown that only the upstream kinase is upregulated under elevated temperatures, whereas the energy sensor remains unaffected. In our study, the alteration of the tight junction proteins in a broiler's jejunum under hyperthermia appeared unrelated to AMPK.

In conclusion, although our understanding of this area of study is far from complete, this study showed that heat stress, as well as AMPK inhibitor injection, effectively impedes the tight junction network. The heat-mediated disturbance of the tight junction network seemed to be independent of the energy sensor. Characterizing an AMPK-independent signaling pathway, the protein dynamics and how these interactions promote or hamper the paracellular barrier tightening might lead to the next level of understanding of tight junction physiology under hyperthermia.

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