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ORIGINAL CONTRIBUTION

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Obesity-related promotion of aberrant crypt foci in DMH-treated obese Zucker rats correlates with dyslipidemia rather than hyperinsulinemia

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Abstract *Background* Obesity and energy restriction modulate the development of precancerous aberrant crypt foci (ACF) in animal models of colon cancer. *Aim* Investigation of the major obesity-associated determinants for ACF-development and underlying mechanisms leading to ACF-modulation, such as changes in DNA damage or colonocytes hyperproliferation. *Methods* Lean and obese Zucker rats fed ad libitum (a.l.) or obese pair fed (p.f.) were induced with 1,2-dimethylhydrazine (DMH) for colon cancer. Multiple regression analyses were performed to identify major metabolic factors correlated with ACF number and size (aberrant crypts/ACF). DNA damage is analyzed by the comet-assay, epithelial proliferation by immunohistochemistry. *Results* Aberrant crypt foci number was significantly elevated in Zucker obese a.l. (205.7 ± 65.4 vs. lean 9.5 ± 6.3, $P < 0.05$) and is reduced by pair feeding in Zucker obese rats (81.4 ± 28.5 vs. obese a.l., $P < 0.05$). Compared to lean the ACF size was higher in Zucker obese a.l. (2.1 ± 0.3 vs. lean 1.3 ± 0.2., $P < 0.05$) but is not reduced by pair feeding (1.7 ± 0.2; $P > 0.05$). While ACF number and size were modulated by genotype and/or pair feeding the DMH-induced DNA damage and hyperproliferation in colonocytes did not differ significantly between groups. Regression analysis showed that plasma parameters associated with lipid-metabolism (triglycerides, cholesterol, malondialdehyde) significantly correlated with the ACF number and size while parameters linked to carbohydrate-metabolism (glucose, insulin) were weaker determinants. *Conclusion* Obesity or pair feeding-associated modulation of ACF correlate with parameters related to lipid-metabolism but is not accompanied by changes in DNA damage and proliferation.

Key words colon cancer – energy restriction – malondialdehyde – proliferation – DNA damage

Introduction

Epidemiological studies consistently suggest that the combination of over-nutrition and physical inactivity leading to obesity (and the central distribution of adiposity) are associated with the elevated colorectal cancer risk [15]. Despite this observational link of obesity and colon cancer, the exact biological mechanisms underlying this association are complex and

not fully understood, but might involve endogenous metabolites which are linked with obesity-associated insulin resistance and chronic inflammation [16].

In recent years insulin resistance formed the core of this hypothesis as it underlies a cluster of metabolic perturbations, including hyperinsulinemia, hyperglycemia, hypertriglyceridemia, increased plasma levels of free fatty acids (FFA) and total cholesterol (Chol), all shown to have a positive correlation with colorectal cancer risk [36, 48]. As deduced from mechanistical studies these factors potentially promote colon carcinogenesis by different mechanisms. The elevated plasma concentrations of insulin, glucose, FFA and triglycerides (TG) associated with insulin resistance might enhance the proliferative state through elevated insulin signaling in the colon as a non-classical insulin target tissue [43]. Further, the increased availability of energy provided by circulating glucose, TG and FFA could selectively promote growth of transformed colonocytes through induction of mitogenic signaling pathways or affecting transcription factors which regulate cell differentiation and the cellular homoeostasis of apoptosis and proliferation pathways [39, 41]. In addition, insulin resistance causes dysregulation of related growth factor systems, e.g. the insulin-like growth factor (IGF) system [14]. As mediated by colonocytes IGF-1 receptor expression, high levels of IGF-1 combined with decreased IGF binding-protein inhibit apoptosis and promote cell-cycle progression leading to an anabolic state which stimulates cellular growth and differentiation [28, 35]. Further adipokines with pro-inflammatory properties, such as leptin and resistin, have been implicated in the pathogenesis of inflammatory bowel disease and the subsequent development of colon cancer [19], whereas adiponectin might have anti-cancer properties through its anti-angiogenic effects [45]. These data clearly show that mechanisms linking obesity via chronic inflammation with an environment supporting the development of colon cancer are complex and involve several mediators which affect the process of cancer initiation and progression at different steps.

Previous animal studies have shown that diet induced [21] and genetically determined obesity [46] lead to an increased number of chemically induced aberrant crypt foci (ACF) [21, 46] and tumors [46] in the colon. This raises the question whether the obesity-mediated increase in ACF (late marker) is the result of enhanced DNA damage (early marker) or hyperproliferative activity in the colonic epithelium (intermediate marker). Since hyperphagia is discussed as an individual risk factor for colon cancer we have used genetically lean and pair fed (p.f.) obese Zucker rats to determine the effect of an increased body weight on the background of similar energy intake upon colon cancer initiation and promotion.

Further, insulin resistance develops in Zucker obese rats independent of energy intake and allows dissociating the impact of body weight and dietary energy intake under similar hyperinsulinemic milieu by comparison of Zucker obese fed ad libitum (a.l.) and Zucker obese p.f. rats. To consider the complexity of obesity-related metabolic and humoral parameters, we have performed multiple regression analysis to selectively identify the metabolic factors correlated with ACF initiation (ACF number) and promotion (ACF size) in the Zucker rat model.

Materials and methods

Animals and experimental protocol

Female Zucker obese rats (fa/fa, $n = 47$) and their lean counterparts (Fa/fa, $n = 24$) were purchased from Charles River Laboratories (Lyon, France) at the age of 5 weeks and housed in a temperature- and humidity-controlled animal unit under ambient temperature of 21 ± 2°C and a 12–12 h light–dark cycle. Due to the described effect of isoflavones on 1,2-dimethylhydrazine (DMH)-induced development of ACF [13], the rodent chow (AIN-93G) was free of soy protein and contained 7% (w/w) of corn oil instead of soy oil [31]. The experimental study design followed a strategy as detailed in Fig. 1. Zucker obese rats were randomly divided into an a.l. fed group ($n = 24$) and a p.f. group ($n = 23$). The group of p.f. rats were fed the amount of food consumed by the age-matched lean counterparts on the previous day resulting in a relative restriction of approx. 30% for energy and all food ingredients when compared to the obese a.l group. Food intake was recorded daily and body weight was recorded four times a week. After 2 weeks of feeding regimen, half of each group was initiated by intraperitoneal injections of DMH (15 mg/kg body wt) and controls received 0.9% NaCl four times at 1-week intervals. Five weeks after the final injection animals were fasted overnight and sacrificed by decapitation. Biological samples were prepared for the respective analytical methods and processed as described below. All animal experiments were performed in accordance with the guidelines of the ethic committee responsible for the district of Karlsruhe (No. 35-9185.81/G-52/05).

Blood parameters

Blood was collected into serum and EDTA monovettes (Sarstedt, Germany) and all blood samples were immediately stored on ice until centrifuged (10 min, 1,500*g*). Total Chol (CHOD-PAP, Roche, Mannheim,

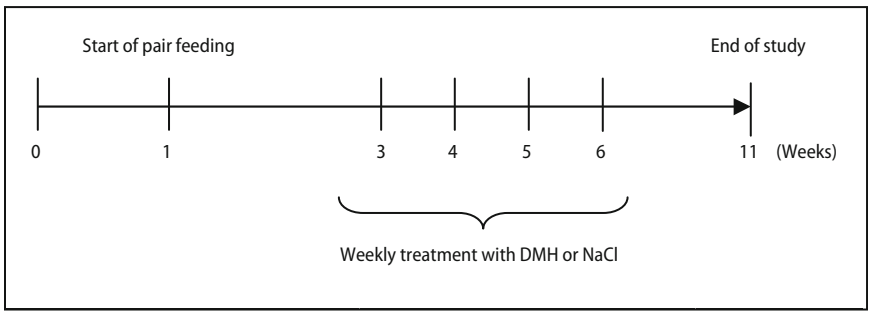

Fig. 1 Experimental design of the study including the animal numbers used for the respective assays. Colonic mucosa of the distal colon was used for all tissue-based assays

Assay	Treatment	*lean* a.l.	*obese* p.f.	*obese* a.l.
Proliferation / plasma parameters	NaCl	6	6	6
ACF / proliferation / plasma parameters	DMH	6	5	6
DNA damage / plasma parameters	NaCl	6	6	6
	DMH	6	6	6

Germany), TG (GPO-PAP, Roche), FFA (FFA C kit, ACS ACOD, Wako, Neuss, Germany) and glucose (Gluco-Quant, Roche; Ecoline 25, Diagnostica, Merck) were determined with the respective kits. Serum insulin (BIOTREND, Köln, Germany), serum leptin and resistin (BioVendor, Heidelberg, Germany), adiponectin (AdipoGen, Axxora, Grünberg, Germany) and IGF-1 (Immunodiagnostic System, Frankfurt, Germany) were analyzed by ELISA kits. Plasma concentration of malondialdehyde (MDA) was determined using a HPLC method previously described by Briviba et al. [8].

Genotyping

A restriction fragment length polymorphism (RFLP)-PCR was used to confirm the genotypes of the respective groups used for the experiments (Fa/Fa, Fa/fa, fa/fa). For isolation of DNA hair roots of the Zucker rats were placed in a centrifuge tube, incubated overnight in lysis buffer at room temperature and samples were further processed according to the manual of the Invisorb Forensic Kit I (Invitek, Berlin-Buch, Germany). DNA was finally resuspended in 50 µl of nuclease free water and a 1 µl aliquot of the isolated DNA was added to 24 µl of PCR reaction volume. The PCR was performed in a thermocycler (PTC 200, MJ Research Inc., Waltham, MA) according to the kit manual (Ready-to-go PCR kit; GE Healthcare, Freiburg, Germany). PCR cycling conditions were: initial denaturation at 95°C for 5 min, amplification over 40 cycles at 95°C for 30 s, 55°C for 20 s, 72°C for 45 s, final extension at 72°C for 6 min. The sequence of the primers was as follows: sense 5′ CTG GGT TTG CGT ATG GAA GT 3′ and antisense 5′ CCT CTC TTA CGA TTG TAG AAT T 3′ (*Rattus norvegicus* leptin receptor, GenBank accession number NM_012596) with a final concentration of 2.5 µM. The PCR product (17.5 µl) was digested with 10 units *MSP I* (New England Biolabs, Frankfurt, Germany) in a 20-µl volume for 3 h at 37°C. The PCR product was loaded on a 1.7% agarose gel, electrophoresis was performed and analysed using an automated computer-based image analysis system (Quantity One/FluorS Imager, Biorad, München, Germany) after staining the gel with ethidiumbromide. The wild type genotype was recognised by a 127 bp band, the homozygote mutant genotype product by 89 and 38 bp band. All three were visible in heterozygotes.

Single cell microgel electrophoresis assay (comet assay)

The entire colon of each animal was isolated immediately after decapitation (n = 6/group) and the distal portion of the colon was used for subsequent analysis by comet assay, proliferation- and ACF-assays as detailed in the respective chapters below (Fig. 1). For

the subsequent procedure the distal colon was placed in pre-warmed buffer (137 mM NaCl, 5.4 mM KCl, 337 μM $Na_2HPO_4 \cdot 2H_2O$, 278 μM $K_2HPO_4 \cdot 3H_2O$, 5.6 mM glucose, 4.2 mM $NaHCO_3$ and 20 mM HEPES). Mucosa cell isolation and determination of genotoxicity was performed according to the procedure described earlier [47]. Three slides with 50 cells were analyzed for each animal using the imaging software of Perceptive Instruments (Halstead, UK). Starting at the center of comet head the percentage of tail intensity was scored reflecting the amount of damaged DNA.

Quantification of ACF and colonic cell proliferation

The bromodeoxyuridine (BrdU) proliferation assay and the detection of ACF were performed according to a recently published protocol [4]. Based on crypt multiplicity (crypts/focus) ACF were further categorized into primal (≤3 crypts/focus) and advanced (≥4 crypts/focus). Crypts were considered aberrant, if they were visibly enlarged and protruding when compared to surrounding crypts, having elongated openings and increased pericryptal zones.

BrdU-labeled cells were visible after immunostaining. Twenty-five well-oriented crypt columns per animal were randomly chosen for counting BrdU-labeled and unlabeled epithelial cells from entire tissue section. Only those crypts were selected with the lumen visible from the bottom to the mucosal surface and with a single layer of cells along each crypt column. BrdU-index was calculated as 100 times the mean number of labeled cells per crypt column divided by the mean total number of cells per crypt column.

Statistical evaluation

Group difference of continuous variables were ascertained by ANOVA using the StatView program (1998; SAS Institute Inc., Cary, NC, USA). Logarithmic, surd or reciprocal surd transformations were performed if the equal variance and normality assumption of ANOVA were rejected. For post hoc comparisons of means, Tukey's honest significant difference test was used. Additionally two stepwise multiple regression analysis was used to identify independent predictors of ACF number as an indicator for initiation of preneoplastic lesions and ACF size which reflects the level of promoting capacity (SAS 1.1). As independent variables besides body weight and energy intake in the first model we used all analysed obesity-linked factors related to carbohydrate metabolism (insulin, glucose, IGF-1) and in the second model factors related to lipid status (TG, FFA, Chol, MDA) and in each case either ACF number or size as dependent variable. Values of $P < 0.05$ were considered significant. All data are presented as the mean ± standard deviation of the mean.

Results

Genotype, food intake, body weight

To assure the genotype before the start of the experiment all animals were genotyped by RFLP-PCR with DNA extracted from hair roots, and 47 homozygote obese fa/fa Zucker rats and 24 heterozygote lean Fa/fa rats were identified (data not shown).

With 7 weeks of age at the beginning of pair feeding obese rats weighed significantly more than lean rats (Table 1). As soon as 3 days after starting the p.f. regimen the mean body weight significantly differed among all three groups which was maintained throughout the entire duration of the experiment. Further pair feeding did not prevent the development of obesity, although the mean rate of weight gain decreased as compared to obese a.l. (Table 1). Energy efficiency was similar in both obese groups and significantly higher compared to lean rats (Table 1).

Endocrine–metabolic profile

A summary of plasma endocrine and metabolic parameters is listed in Table 2. None of these parameters was affected by DMH treatment. From these data plasma parameters which solely depend on the genotype can be distinguished from those which depend on both genotype and energy intake or which were not affected by either genotype or energy intake.

Table 1 Food intake, body weight and weight gain in lean and obese Zucker rats fed ad libitum or pair fed[a]

	Lean ($n = 24$)	Obese pair fed ($n = 23$)	Obese ad libitum ($n = 24$)
7-week body weight (g)	125.6 ± 7.0 x	159.1 ± 14.2 y	165.3 ± 3.1 y
15-week body weight (g)	248 ± 12.6 x	328.6 ± 20.08 y	409.5 ± 33.6 z
Food intake (g/day)	13.7 ± 0.8 x	13.6 ± 0.0 x	19.4 ± 1.3 y
Weight gain (g/week)	13.4 ± 1.34 x	18.1 ± 2.6 y	26.9 ± 2.9 z
Energy efficiency (g gain/MJ intake)	8.6 ± 0.7 x	11.3 ± 1.9 y	11.5 ± 1.1 y

[a]Data are the means ± SD. Values in a common row with different letters are significantly different ($P < 0.05$)

Table 2 Biochemical blood parameters in lean and obese Zucker rats fed ad libitum or pair fed[a]

	Lean (n = 24)	Obese pair fed (n = 23)	Obese ad libitum (n = 24)
Cholesterol (mg/dl)	79.73 ± 17.0 x	315.4 ± 35.9 y	340.0 ± 40.1 y
FFA (mmol/l)	0.8 ± 0.2 x	1.0 ± 0.3 y	1.4 ± 0.4 z
Triglycerides (mg/dl)	55.8 ± 14.2 x	137.6 ± 58.1 y	366.0 ± 289.4 z
Glucose (mg/dl)	104.8 ± 9.9 x	223.8 ± 130.8 y	140.3 ± 25.9 y
Insulin (ng/ml)	0.5 ± 0.1 x	42.5 ± 33.7 y	23.0 ± 17.8 y
IGF-1 (ng/ml)	1025.8 ± 184.6 x	671.3 ± 207.3 y	992.0 ± 172.9 x
Resistin (ng/ml)	10.2 ± 2.2 x	12.3 ± 2.6 y	9.5 ± 2.4 x
Adiponectin (μg/ml)	6.0 ± 0.8 x	6.9 ± 2.3 x	5.8 ± 1.0 x
Leptin (ng/ml)	2.4 ± 1.0 x	66.1 ± 15.3 y	71.1 ± 13.2 y
Malondialdehyde (μM)	1.4 ± 0.3 x	3.0 ± 0.5 y	4.3 ± 1.1 z

[a]Data are the means ± SD. Values in a common row with different letters are significantly different ($P < 0.05$)

When compared with the lean group Chol plasma concentrations as well as glucose and insulin were significantly elevated in obese rats and not further modulated by the level of dietary energy intake. Further, the plasma concentration of FFA, TG and MDA was significantly elevated by the obese genotype and further increased significantly by hyperphagia in the obese group fed a.l. While plasma adiponectin levels did not significantly differ between groups, plasma IGF-1 significantly decreased and plasma resistin significantly increased in the p.f. obese group when compared to the lean and the obese a.l. groups.

Genotoxicity

To determine whether energy intake or the obese genotype significantly affected the level of DNA damage in the colon mucosa a comet assay was performed. As shown in Table 3, the treatment with DMH significantly increased the DNA damage in colon mucosa cells when compared to the respective control groups receiving NaCl instead of DMH. Further, neither the genotype nor the energy intake significantly affected mucosal DNA damage in DMH or NaCl groups (Table 3).

Epithelial proliferation

In all animals analyzed, BrdU-labeled cells were exclusively found in the lower part of the crypt. Compared with the respective controls receiving NaCl, DMH did not modulate the epithelial proliferation. Neither the genotype nor the pair feeding significantly affected the proliferation index in colon crypts in both DMH and NaCl groups. However, data analysis showed a trend within the DMH-treated groups for a higher proliferation rate of the obese rats fed a.l. as compared with the lean rats ($P = 0.03$, Fisher Test, Fig. 2).

Table 3 DNA strand breaks in mucosa cells of the distal colon[a]

	Mean tail intensity (%)		
	Lean	Obese pair fed	Obese ad libitum
NaCl	3.2 ± 1.1 x	3.0 ± 0.7 x	4.1 ± 1.3 x
DMH	8.0 ± 1.0 y	8.0 ± 1.6 y	9.2 ± 1.3 y

[a]Data are the means ± SD (n = 6 animals/group). Values in a common row and column with different letters are significantly different ($P < 0.05$)

Aberrant crypt foci

The ACF number was significantly higher in obese groups compared to lean and was significantly reduced by pair feeding (Fig. 3a). On the other side the ACF size, representing an indicator for the promoting capacity, was significantly elevated to an equal level in obese p.f. and a.l. rats compared to lean rats (Fig. 3b). Also the number of primal and advanced ACF was elevated in obese rats, but did not change significantly in the p.f. rats as compared to the a.l. group (Fig. 3c).

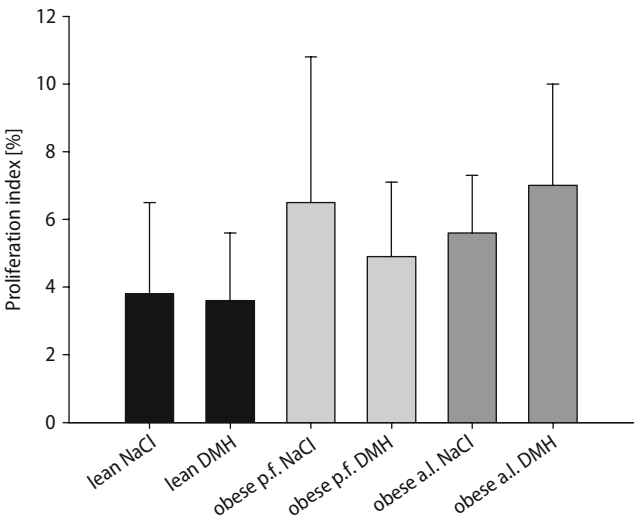

Fig. 2 Proliferation index in the distal colon as the percentage of BrdU-positive cells determined within 25 randomly chosen crypts per animal. *Bars* represent means ± SD

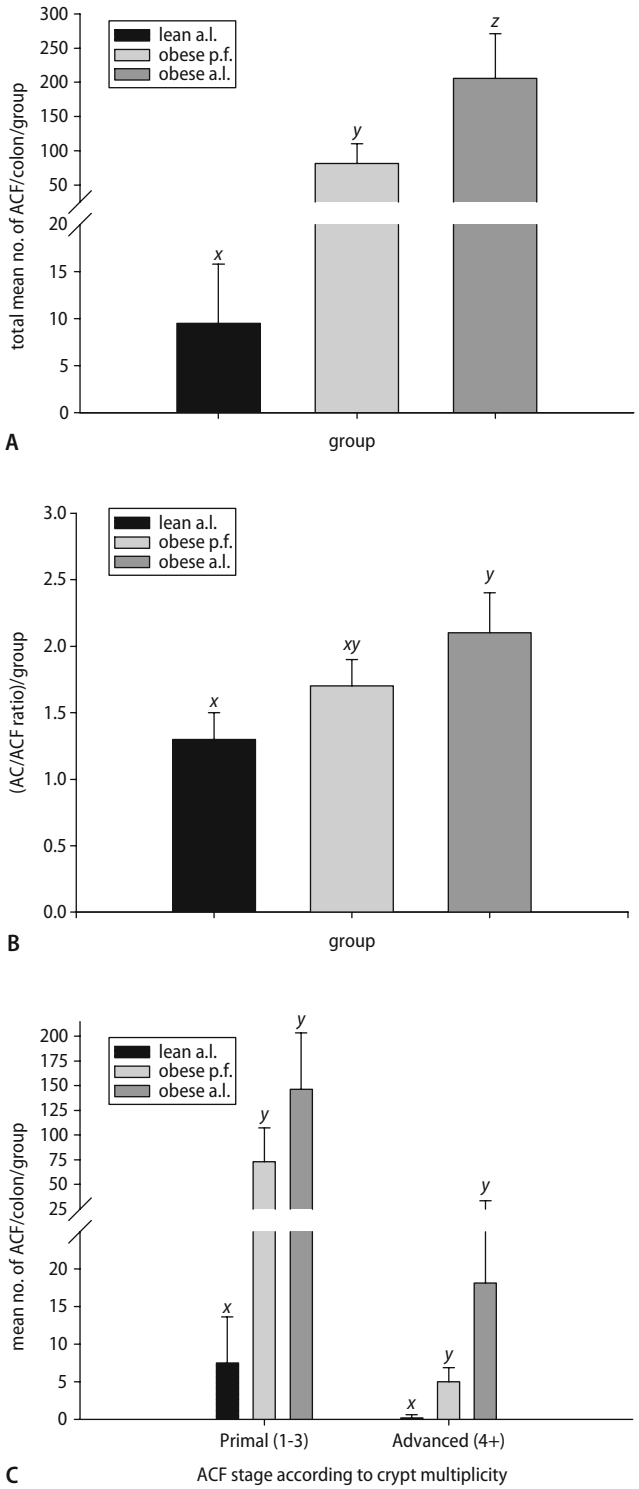

Fig. 3 Analysis of aberrant crypt foci (*ACF*) total number (**A**) and size (**B**) and the number of primal (1–3 crypts) and advanced ACF (4 or more crypts) (**C**) in the distal colon mucosa of DMH-treated animals. *Bars* represent means ± SD. Values with different *superscripts* are significantly different, $P < 0.05$

Stepwise regression analysis

To determine specific metabolic and endocrine factors associated with obesity that further correlate well with number and size of ACF a stepwise multiple regression analysis has been conducted involving either Chol, MDA, TG, FFA or insulin, IGF-1 and glucose as independent variables. In addition, the general parameters, body weight or energy intake, were combined with both models. As a single measure all parameters except of FFA, IGF-1 and glucose significantly correlated with the number of ACF and in a weaker manner also with ACF size (Table 4). In the first model energy intake, body weight and Chol correlated with ACF number, and Chol and body weight with ACF size (Table 5). For the ACF number the correlation with carbohydrate-related parameters was not significant as in that model energy intake and body weight were the only factors correlated with ACF number. Regarding ACF size the correlation with body weight and energy intake was weaker whereas the correlation with insulin and glucose was stronger if compared to ACF number (see Table 6). Beside these most dominant predictors (body weight, energy intake and Chol) MDA was the strongest determinant of ACF number but not of ACF size. The MDA plasma concentration significantly correlated with TG and body weight (R^2 adj. = 0.7543; TG $P = 0.0002$, body weight $P = 0.0018$) but not with FFA or energy intake (FFA $P = 0.7139$, energy intake $P = 0.6119$).

Discussion

The observation that obese rats are more sensitive to colon carcinogenesis compared to their lean counterparts has been investigated in different animal models of obesity [29]. In our study we could confirm these data as hyperphagic obese Zucker rats developed a higher number and size (AC/ACF) of ACF. Additionally, we could show that pair feeding, which resulted in a relative restriction of 30% for energy and total food ingredients, led to a substantial decrease in the total number of ACF in obese, but did not lower the ACF size significantly. Further, the major aim of the present study was to determine whether this elevation of ACF (late marker) is the result of increased DNA damage (early marker) or hyperproliferative activity in the colon epithelium (intermediate marker). In this context we could show that the modulation of ACF number by the obese genotype (body weight) and by the amount of energy intake was not paralleled by concomitant changes in DNA damage or proliferation rate. From these results the question

Table 4 Regression analysis with ACF number and ACF size as dependent variables

Independent variable for simple regression	ACF number				ACF size			
	Parameter estimate	Standardized estimate	*P*	R^2 adj.	Parameter estimate	Standardized estimate	*P*	R^2 adj.
Body weight (g)	0.139	0.880	<0.0001	0.760	0.004	0.748	0.0006	0.530
Energy/week (MJ/w)	210.3	0.877	<0.0001	0.755	0.685	0.684	0.0024	0.433
Cholesterol (mg/dl)	0.688	0.827	<0.0001	0.662	0.003	0.788	0.0002	0.596
MDA (μM)	73.79	0.791	0.0002	0.600	0.246	0.633	0.0064	0.361
TG (mg/dl)	0.645	0.691	0.0021	0.443	0.002	0.623	0.0075	0.348
FFA (mmol/l)	120.6	0.419	0.0935	0.121	0.313	0.261	0.3112	0.006
Insulin (ng/ml)	1.809	0.516	0.0340	0.217	0.008	0.575	0.0157	0.286
IGF-1 (ng/ml)	0.011	0.029	0.9098	−0.066	0.000	0.040	0.8764	−0.065
Glucose (mmol/l)	0.187	0.180	0.4880	−0.032	0.000	0.024	0.9241	−0.066

Table 5 Stepwise multiple regression analysis with ACF number and size as dependent variables

Independent variable for regression						ACF number				ACF size			
C (mg/dl)	*E* (MJ/w)	MDA (μM)	TG (mg/dl)	FFA (mmol/l)	BW (g)	Parameter estimate	Standardized estimate	*P*	R^2 adj.	Parameter estimate	Standardized estimate	*P*	R^2 adj.
•	•					141.2 *E* 0.369 *C*	0.589 *E* 0.443 *C*	0.0002 *E* 0.0025 *C*	0.866	0.002 *C*	0.595 *C*	0.0106 *C*	0.625
•		•				0.447 *C*	0.537 *C*	0.0317 *C*	0.695	0.003 *C*	0.767 *C*	0.0126 *C*	0.568
•			•			0.536 *C*	0.6439 *C*	0.0020 *C*	0.705	0.002 *C*	0.649 *C*	0.0053 *C*	0.606
				•	•	0.947 BW	0.834 BW	<0.0001 BW	0.763	0.004 BW	0.745 BW	<0.0014 BW	0.497
•	•			•		136.4 *E* 0.346 *C*	0.569 *E* 0.416 *C*	0.0003 *E* 0.0038 *C*	0.875	0.002 *C*	0.596 *C*	0.0148 *C*	0.596
•		•	•			0.447 *C*	0.536 *C*	0.0333 *C*	0.695	0.003 *C*	0.766 *C*	0.0115 *C*	0.592
•	•	•		•		165.3 *E* 0.443 *C*	0.690 *E* 0.532 *C*	0.0004 *E* 0.0027 *C*	0.883	0.003 *C*	0.753 *C*	0.0141 *C*	0.597
		•	•	•		58.78 MDA	0.630 MDA	0.029 MDA	0.563				

Stepwise multiple regression analysis involving cholesterol (*C*), energy intake/week (*E*), malondialdehyd (MDA), triglycerides (TG), free fatty acids (FFA) and body weight (BW) as independent variables was performed, and ACF number and size were individually analyzed as a dependent variable. Only significant variables are shown ($P < 0.05$)

arises about the mechanistic origin of changes in ACF number and/or size by obesity and pair feeding.

As the degree of DNA damage seems to be independent of the obese genotype and the amount of energy intake, mechanistic artefacts could be excluded as the origin for variations in ACF. It is well known, that DMH is mainly metabolized by P4502E1 and exerts its carcinogenic effect by increasing the main DMH adduct O^6-methylguanine [33]. The hypothesis that energy restriction (ER) and obesity alter cytochrome P4502E1 and by this way modulate the biotransformation rates of DMH thus influencing its carcinogenetic potential [23, 30] could not be confirmed in the present study as DNA strand breaks did not differ between groups.

Further, the altered number of ACF was not related to the proliferative status of the epithelium during the initiation phase. Previous studies have already reported that DMH-treatment results only in a temporary hyperproliferation [17, 32]. Further it has already been shown that mucosal hyperplasia has a profound effect upon initiation of carcinogenesis [5], and little effect upon existing neoplasia [6], possibly explaining the absence of hyperproliferation by the observed concomitantly higher number of ACF in the obese Zucker rat.

Raju et al. [29] have shown that ER performed in frame of a post-initiation protocol (starting 1 week after the final of two weekly AOM injections) inhibits the outgrowth from early to advanced ACF rather than their persistence which might lead to the suggestion, that early and advanced ACF may respond differently to growth modulation [7, 21]. Operational reversibility is a principal characteristic of the promotion phase [26] as underlying mechanisms may implicate cell death, apoptosis [44] regression or remodeling [7]. It has been observed that ER augments apoptosis [11, 27] and might therefore be one explanation how ER modulates the number of ACF without affecting the proliferative capacity.

Recently many studies have suggested that hyperinsulinemia might underlie the observed positive correlation of metabolic syndrome and colon carcinogenesis, since insulin can directly or indirectly stimulate the growth of colonic epithelium [43]. However, our results of multiple regression analysis

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Table 6 Stepwise multiple regression analysis with ACF number and size as dependent variables

Independent variable for regression					ACF number				ACF size			
I (ng/ml)	*E* (MJ/w)	IGF-1 (ng/ml)	*G* (mmol/l)	BW (g)	Parameter estimate	Standardized estimate	*P*	R^2 adj.	Parameter estimate	Standardized estimate	*P*	R^2 adj.
•			•		2.320 *I*	0.662 *I*	0.0367 *I*	0.199	−0.002 *G*	−0.550 *G*	0.0369 *G*	0.446
									0.014 *I*	0.919 *I*	0.0018 *I*	
		•		•	1.013 BW	0.892 BW	<0.0001	0.752	0.004 BW	0.756 BW	0.0008 BW	0.500
•		•	•		2.316 *I*	0.661 *I*	0.0541 *I*	0.138	−0.003 *G*	−0.702 *G*	0.0370 *G*	0.434
									0.014 *I*	0.983 *I*	0.0019 *I*	
•	•	•			209.6 *E*	0.874 *E*	<0.0001 *E*	0.859	0.601 *E*	0.601 *E*	0.0071 *E*	0.551
					0.842 *I*	0.240 *I*	0.034 *I*		0.006 *I*	0.395 *I*	0.0482 *I*	
•		•	•	•	1.016 BW	0.895 BW	0.0002 BW	0.711	0.003 *E*	0.527 *E*	0.0223 *E*	0.610
									−0.003 *G*	−0.553 *G*	0.0527 *G*	
									0.009 *I*	0.586 *I*	0.0427 *I*	
•	•	•	•		210.790 *E*	0.880 *E*	<0.0001 *E*	0.847	0.521 *E*	0.521 *E*	0.0090 *E*	0.660
									−0.042 *G*	−0.545 *G*	0.0417 *G*	
									0.011 *I*	0.722 *I*	0.0054 *I*	

Stepwise multiple regression analysis involving insulin (*I*), energy intake/week (*E*), IGF-1, glucose (*G*) and body weight (BW) as independent variables was performed, and ACF number and size were individually analyzed as a dependent variable. Only significant variables are shown ($P < 0.05$)

indicate that Chol and MDA rather than insulin and glucose are the major plasma parameters which may be involved in the steps of colon carcinogenesis. Further we could show that the clustering of these components has a higher correlation coefficient with ACF compared with the individual parameters suggesting that these components promote the development of ACF via different mechanisms.

As confirmed in our study, obese rats showed significantly increased leptin plasma levels even after a significant energy restriction by the pair feeding strategy. The role of leptin in carcinogenesis is controversially discussed, as leptin either enhanced colon cancer [46] or did not modulate AOM-induced ACF [2, 3]. Under conditions of leptin receptor deficiency a direct role for leptin in modulating DMH-induced ACF in the Zucker rat could be excluded. However, leptin resistance determines the obesity mediated glucose intolerance and hyperinsulinemia [20] and is one factor that contributes to obesity-associated insulin resistance [25] as also observed in the Zucker rat.

It has been hypothesized that at least some of the protective properties of dietary ER on colon cancer are due to the modulation of hormonal and metabolic profiles which are disarrayed in the obese state [22]. The assessment of blood levels of insulin, glucose and Chol confirmed the possible interrelation between the disarrayed hormonal or metabolic parameters encountered in obesity but pair feeding did not change the levels of these parameters. The variances of mean plasma glucose and insulin levels in the obese p.f. groups are larger than in the lean and obese a.l. groups which might explain the lacking statistical significance comparing obese p.f. and a.l. groups.

The obese Zucker rats did not show increased total IGF-1 plasma levels compared to lean rats but pair feeding significantly reduced total IGF-1. The fact that obesity has a more important influence on the amount of IGF-1 available to cells [12] than on total circulation IGF-1 or IGFBP-3 [42] would explain that total circulating IGF-1 was not altered in obese Zucker rats compared to lean rats. A decline of IGF-1 by pair feeding was shown in animals [34] and humans [38]. There is some evidence that changes in IGF-1 levels may be relevant for the effects of caloric restriction on tumor formation [11].

Fasting plasma FFA levels were almost twice as high in obese than in lean rats confirming earlier results of Abadie et al. [1] and pair feeding significantly reduced FFA. FFA interferes with insulin signal transduction and promote inflammation and oxidative stress. This may lead to insulin resistance in liver and skeletal-muscle [9]. Further, FFA may provide increased energy for transformed colonocytes as well as induce changes in cell signaling pathways.

MDA and TG showed the same plasma patterns as FFA with lowest plasma concentrations in lean, intermediate in p.f. and the highest in obese a.l. fed animals. Regression analyses indicate that MDA changes are mainly explained by TG apart from body weight.

On the one hand MDA serves as a biomarker for oxidative stress [18] and on the other hand may be also involved in tumor promotion itself because it can interact with functional groups of a variety of cellular compounds, including the amino-groups of proteins and nucleic acid bases, the N-bases of phospholipids and the SH-groups of sulphydryl compounds [24, 37]. So the reduction of MDA in the p.f. rats compared to the a.l. fed group is closely linked to circulating TG and indicates reduced lipid peroxidation with the consequence of reduced exposure of, e.g. DNA to MDA. The reduction of TG by pair feeding in the obese group may result from lower intake of exogenous energy, a reduction of body weight and FFA by pair feeding probably causing a reduced synthesis and secretion of hepatic lipoprotein. Other underlying mechanisms could also be a modulation of the level of lipolysis and the clearance of TG.

Hypertriglyceridemia itself has also been associated with colorectal adenoma and carcinoma risk [40, 48]. According to their plasma patterns and their role in carcinogenesis IGF-1, FFA, MDA and TG might be the major factors contributing to the lower number of ACF in p.f. rats.

In summary the obese genotype resulted in a remarkably higher sensitivity to DMH-induced ACF compared to the lean genotype. Pair feeding reduced the body weight, however could not prevent the outcome of obesity but nonetheless seems to be a potent protective factor, reducing the total number of ACF. Zucker obese rats responded to relative energy restriction by pair feeding in presence of the hyperinsulinemic state arguing against a direct relationship between insulin levels and early stages of colon cancer, which has been lately the main hypothesis represented [14]. This suggestion is in line with recent findings which did not support a direct link between deregulation of insulin levels and increased risk of colon carcinogenesis in obese rats [10]. In consideration that the results could be confined only to the model of the Zucker rat, we suggest that TG, FFA, MDA and IGF-1 are associated with the pair feeding-triggered effects on carcinogenesis leading to a reduced number of ACF which is not associated with changes in DNA damage and colonic epithelial proliferation.

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