Supporting Information

Low-dose naltrexone rescues inflammation and insulin resistance associated with hyperinsulinemia

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Running title: LDN protects against systemic inflammation and insulin resistance.

Keywords: Low-dose Naltrexone, SIRT1, Inflammation, NF-κB, Hyperinsulinemia, Insulin resistance, Toll-Like Receptor 4

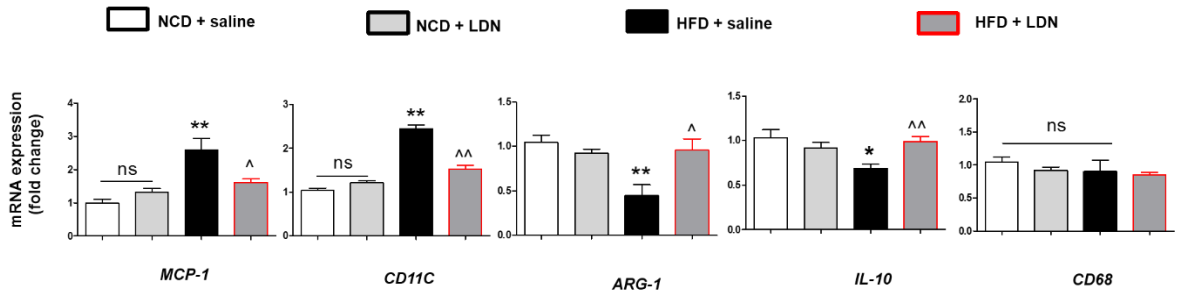

Figure S1: Effect of LDN on hyperinsulinemia induced inflammatory genes expression: Quantitative mRNA expression of indicated genes in diet induced hyperinsulinemic mice. Values are expressed as mean ± SD. **P<0.01, *P<0.05 NCD+saline and ^^P<0.01, ^P<0.05 HFD+saline. (ANOVA followed by Bonferroni's Multiple Comparison).

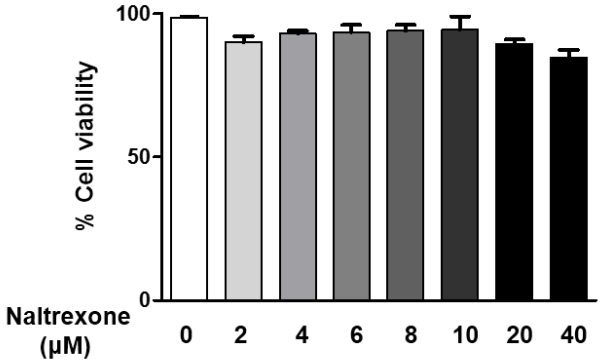

Figure- S2: Effect of naltrexone on Raw264.7 cells viability. Raw 264.7 (macrophage) cells were exposed to different doses of naltrexone for 24 hours (hr) and MTT assay were performed to determine the viability. Viability was represented as percentage (%) as relative to control. All data were presented as Mean ± SD of three independent sets of experiments.

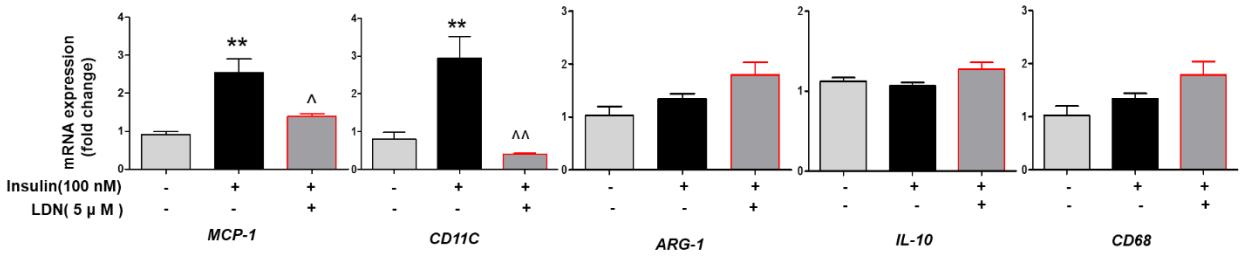

Figure- S3: Effect of LDN on hyperinsulinemia induced inflammatory genes expression in *in-vitro*. Macrophage cells were challenged with 100 nM insulin with or without LDN for 24 hr and indicated inflammatory modulatory genes were analysed. Values are expressed as mean ± SD. **P<0.01, *P<0.05 Vs Control and ^^P<0.01, ^P<0.05 Vs Insulin. (ANOVA followed by Bonferroni's Multiple Comparison).

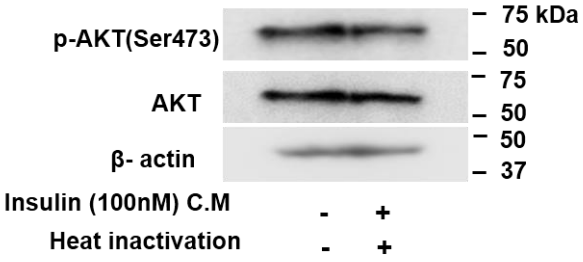

Figure- S4: Immunoblot for p-AKT(Ser473) in HepG2 cell lysate treated with indicated conditioned media (C.M), after heat inactivation.

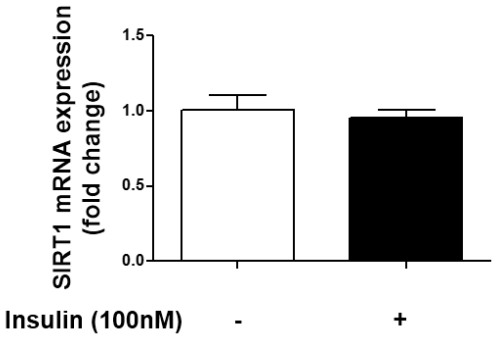

Figure- S5: Quantitative mRNA expression of Sirt1 mRNA levels in cells treated with or without insulin for 18 hr . Values are expressed as mean ± SD of three independent sets of experiments.

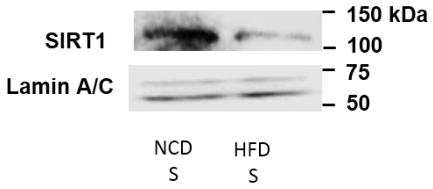

Figure S6: Hyperinsulinemia downregulates SIRT1 protein. IB (Immunoblot) representing nuclear SIRT1 level in white adipose tissue lysate from NCD-saline (NCD S) and HFD-saline (HFD S) group of mice.

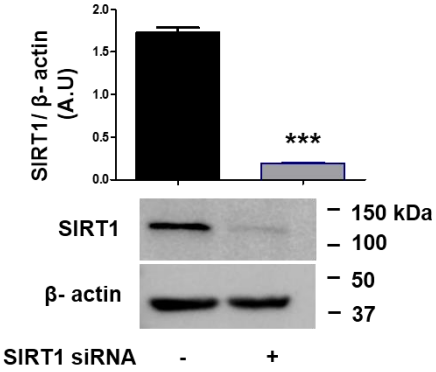

Figure S7: SIRT1 knockdown in Raw264.7 cells. Representative immunoblot of macrophage cell lysates shows 90% reduction of SIRT1 protein 48 hr after siRNA transfection and densitometric analysis of immunoblot. Values are expressed as mean ± SD of three independent sets of experiments.

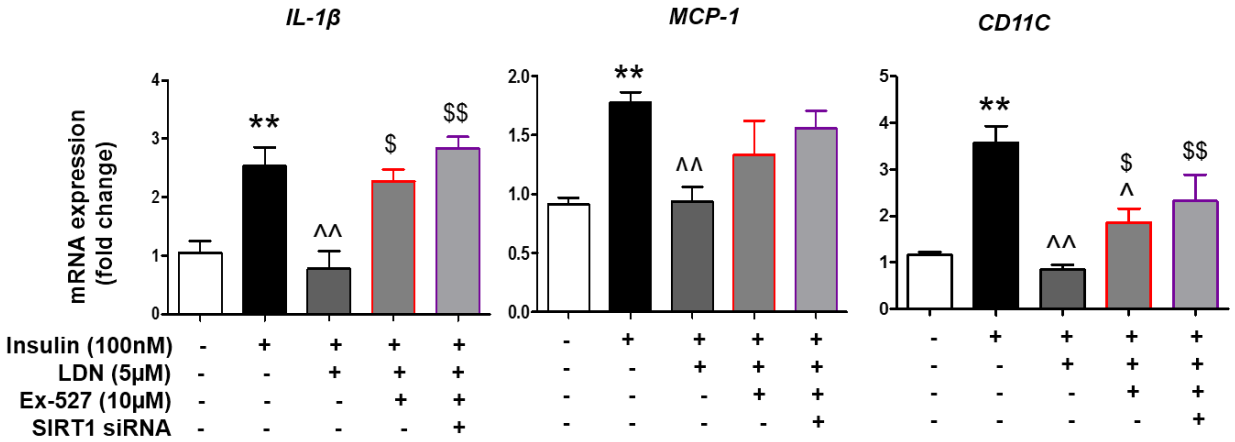

Figure S8: LDN stimulated anti-inflammatory phenotype depends on SIRT1. Macrophage cells were incubated with or without 100 nM insulin for 24 hr in the presence and absence of 10μM EX-527 or Sirt1 siRNA with indicated conditions and quantitative mRNA levels of inflammatory genes were analyzed. Values are expressed as mean ± SD. **P<0.01, *P<0.05, Vs Control, ^^P<0.01, ^P<0.05, Vs Insulin and $ P<0.05, $$ P<0.01 Vs Insulin-LDN. (ANOVA followed by Bonferroni's Multiple Comparison).

Table1: Antibody list

S.No.	**Antibody**	**Species-specific**	**Company**	**Cat No.**
1.	p-AKT(Ser473)	Rabbit	CST	4058
2	p-AKT (Thr308)	Rabbit	CST	4056
3.	AKT	Rabbit	CST	9272
4	p-GSK3β (Ser9)	Rabbit	CST	9323
5	GSK3β	Rabbit	CST	9315
6.	SIRT1	Mouse	CST	2028
4.	β-actin	Rabbit	CST	4970
5.	β-actin	Mouse	Santa Cruz Biotechnology	sc-47778
6.	p-NF-κB P65(Ser536)	Rabbit	CST	3033
7.	NF-κB P65	Mouse	CST	6956
8.	Anti-rabbit (secondary antibody)	Rabbit	CST	7074
9.	Anti-mouse (secondary antibody)	Mouse	CST	7076

Table 2: Primer sequences

S.No.	**Gene**	**Forward primer (5'-3')**	**Reverse primer (3'-5')**
1.	MCP-1	GAAGGAATGGGTCCAGACAT	ACGGGTCAACTTCACATTCA
2.	IL-1β	CACAGCAGCACATCAACAAG	GTGCTCATGTCCTCATCCTG
3.	CD11c	ATGGAGCCTCAAGACAGGAC	GGATCTGGGATGCTGAAATC
4.	TLR-4	CAATCGCATAGAGACATA	GTTCAACATTCACCAAGA
5.	TNF-α	TCTTCTCATTCCTGCTTGTGG	GGTCTGGGCCATAGAACTGA
6.	IL-6	CTCTGGGAAATCGTGGAAAT	CCAGTTTGGTAGCATCCATC
7	IL-10	ATAACTGCACCCACTTCCCA	GGGCATCACTTCTACCAGGT
5.	ARG-1	TTTTTCCAGCAGACCAGCTT	AGAGATTATCGGAGCGCCTT
6.	CD68	TTGCTAGGACCGCTTATA	AAGGATGGCAGGAGAGTA
7.	18-S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

Table 2: siRNA sequences

Name	**Sequence**	**Species-specific**	**Company**	**Cat No.**
SIRT1	UCCGUAUCAUCUUCCAAGCca	Mouse	AMBION	s96764
SIRT1	UAAUUUCGAAGUAGUUUUCcct	Mouse	AMBION	s96765